Domitília da Conceição Coutinha Matias

Licenciada em Biologia Marinha e Pescas

Bases Biológicas e Ambientes para a Optimização da Produção de Amêijoa-boa *Ruditapes decussatus* (Linnaeus, 1758)

Dissertação para obtenção do Grau de Doutor em Ambiente

Orientador: Paula Sobral, Prof. Auxiliar, FCT-UNL

Co-orientador: Alexandra Leitão, Inv. Auxiliar, IPMA, I.P.

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Presidente: Prof. Doutor José Paulo Barbosa Mota

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Establishment of Environmental and Biological Bases to Optimise the Production of the European Clam Ruditapes decussatus (Linnaeus, 1758)

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“Most of the fundamental ideas of science are essentially simple, and may, as a rule, be expressed in a language comprehensible to everyone.”

Albert Einstein

To Margarida, Xavier and my parents.

Acknowledgements

Finally, the anticipated, yet most anxious, moment of my PhD has arrived, on the one hand, to relief of finishing this thesis, on the other hand the question what will happen next.

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- To everyone who contributed somehow for this study;

- Finally, I can answer all those who were constantly asking the boring question: “So, when will you finish writing the PhD thesis?...” with “It is done” and I promise never to do the same to those who are still rushing to conclude their thesis...

Thank you all...

Resumo

Em Portugal, o sector da aquacultura é grandemente suportado pela produção de *Ruditapes decussatus*, que representa 80 % da produção de moluscos bivalves. No entanto, a cultura de *R. decussatus* encontra-se condicionada pela disponibilidade de semente, uma vez que esta produção é efectuada exclusivamente com base em juvenis provenientes de recrutamento natural. Nos últimos anos, a produção desta espécie tem claramente decrescido devido a falhas de recrutamento e a mortalidades elevadas. O desenvolvimento de técnicas de produção artificial constituirá uma fonte alternativa de juvenis que permitirá ultrapassar estes constrangimentos à produção. A produção de semente de *R. decussatus* em maternidade é uma actividade relativamente recente e baseada em conhecimentos empíricos. Assim, devido à escassez de informação sobre as técnicas de produção de *R. decussatus*, o principal objectivo desta tese consistiu na avaliação dos processos biológicos e ecológicos implicados nas diferentes fases de cultura (acondicionamento de reprodutores, cultura larvar e engorda). Visando a optimização do acondicionamento de reprodutores de *R. decussatus*, caracterizou-se o ciclo reprodutivo das duas principais populações portuguesas, bem como a estratégia de armazenamento e consumo dos constituintes bioquímicos de reserva. Foi identificada qual a melhor proveniência e época de recolha dos progenitores e identificadas as melhores temperatura e dieta a serem usadas no acondicionamento visando maximizar o processo de maturação, o sucesso da postura e a viabilidade larvar. No que concerne a optimização da fase larvar, foi obtida informação essencial sobre a utilização de energia durante a oogénese e metamorfose, bem como qual a melhor dieta a fornecer na fase larvar. Relativamente à fase de engorda, foi avaliada a resposta de juvenis de *R. decussatus* às variações ambientais locais e sazonais, visando a definição de estratégias de gestão da produção. Finalmente, a globalidade da informação obtida no presente estudo contribuiu para o desenvolvimento de um plano eficaz de produção.

Palavras-chave: *Ruditapes decussatus*, Bivalves, Produção artificial, Ciclo reprodutivo, Necessidades nutricionais, Programas de produção.

Abstract

In Portugal, aquaculture is greatly supported by the production of *Ruditapes decussatus* that represents 80 % of the total shellfish production. However, the culture of *R. decussatus* is clearly limited by the availability of seed, which proceeds exclusively from natural recruitment. In the last few years, the production of this species has clearly decreased due to recruitment failures and to severe mortalities. The development of hatchery technology that will provide an alternative reliable source of clam spat, will allow to overcome this constrain. Production of *R. decussatus* seed in hatcheries is however a relatively new industry for which most methods have been developed using empirical approaches. Due to the scarcity of information on this species production, the main objective of the present work was the evaluation of the biological and ecological processes involved in the different culture phases (broodstock conditioning, larval culture and on-growing). Concerning broodstock conditioning, the reproductive cycle of the two main Portuguese populations of *R. decussatus*, as well as its nutrient storage and exploitation strategy were characterized. Moreover, the best broodstock origin and timing of collection were determined, as well as the most adequate conditioning temperature and food to achieve maturation, spawning success and larval viability, limiting factors for the reproduction of this species. In order to optimize the larval management, important information on the energetic utilization during oogenesis and metamorphosis was obtained, contributing to the design of the most suitable diet for the larval phase. Concerning the on-growing phase, the response of *R. decussatus* to local and seasonal environmental factors was evaluated, aiming to define ecological and production management strategies. Finally, the overall information gathered in the present study allowed the development of a successful *R. decussatus* production program.

Keywords: *Ruditapes decussatus*, Bivalves, Hatchery production, Reproductive cycle, Nutritional requirements, Production programs.

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Chapter 1

Introduction

1.1. General Introduction

The production of bivalve molluscs is a strategic activity since it contributes significantly to the preservation of local economies and generates capital and employment on the littoral areas. In Portugal, the production of bivalves is one of the most important social and economic activities, with a great growing potential as a fisheries subsector, due to the edaphic-climatic and geographic conditions. Artisanal production of bivalve molluscs is mainly based on the culture of the European clam (*Ruditapes decussatus*) and oysters (*Crassostrea spp*). *R. decussatus* represents 27 % of the national annual aquaculture production and 64 % of shellfish production (DGPA, 2009). The main production areas of this species are the Ria de Aveiro (40º42'N 08º40'W), the Ria de Alvor (37º07'N 08º36'W) and the Ria Formosa Lagoon (36° 59' N 7° 55'W). The culture of *R. decussatus* in Ria Formosa Lagoon representing 90 % of the national production and it is central to the socioeconomic framework involving, directly or indirectly more than 4500 people (INE, 2007). *R. decussatus* is reared in plots in the intertidal zone exploited by clam farmers, usually organized in professional associations. Clam farmers pay an annual rent to the state, which gives them the exclusive right to cultivate clams on the leasehold. In 2011, there were 1297 licensed clam ground plots within the intertidal area of the Ria Formosa Lagoon, covering a total of 460.10 ha (DGPA, institutional communication). In addition to the official catch figures, widespread illegal and largely opportunistic fishing and harvesting by elements foreign to the local associative system most probably doubles the official production estimates. The Ria de Alvor has 32 licensed ground plots and the Ria de Aveiro has 46 licences that occupy a total of 24 hectares (DGPA, institutional communication). The European clam production still relies on the collection of wild seed. Although this traditional practice has allowed the development of *R. decussatus* production, seed collection can have a negative environmental impact or be a limiting factor for the development of this activity. Indeed, since the two last decades, the European clam production has suffered a decrease due to several constrains, namely recruitment failures and excessive pressure on the capture of juvenile on natural banks, and abnormal mortalities associated to environmental degradation and pathogens (*e.g. Perkinsus olseni*) (Matias, 2007).

The bivalve aquaculture industry depends indeed on the availability of high quality juveniles, which will grow rapidly to commercial size (Ojea et al., 2004). The advantages of hatchery bivalve seed production are numerous. Bivalve hatchery represents a source of high quality juveniles, which does not depend on environmental conditions and the fluctuations of the natural populations' recruitments. It also permits to select the size and weight of seed most suitable to initiate the growth production phase during all year long. When the zootechnical conditions are precisely controlled, it becomes possible to carry out a culture with a precise control of the diet and the environmental conditions, attaining optimum growth rates and healthy

juveniles. The control of the zootecnhical conditions allows the production of juveniles' with certain features of interest such as optimum survival and growth, disease resistance, etc. In this sense, the artificial production of bivalve juveniles appears as a way to satisfy the needs of the aquaculture industry, since it will allow obtaining a controlled product. For hatcheries to consistently produce spat it is essential to develop broodstock conditioning techniques (Lannan et al., 1980; Pronker et al., 2008). To know and manipulate the natural gonadal cycle and spawning period of the clams so that adults can be induced to spawn earlier or later than in the natural environment (Ojea et al., 2008) is crucial. Despite the on-growing know-how in bivalves hatchery, some biological aspects remain unknown or poorly understood, such as bivalve feeding requirements, being the larval stages the most critical stages in the life cycle of bivalves, (Helm et al., 2004; Rico-Villa et al., 2006). Optimizing conditions in the hatchery is, therefore, an important task. The importance of optimizing larvae nutrition is a crucial aspect of overall hatchery operations (Widdows, 1991). Food quantity and food quality are both important in larval cohort success (Powell et al., 2004) and knowledge of the dynamic utilization of the energy reserves stored during endotrophic and exotrophic phase give useful information on the nutritional requirement of larvae (Labarta et al., 1999; Pernet et al., 2004).

The on-growing phase of bivalve aquaculture industry depends also on the availability of ground plots with adequate environmental conditions to organisms' growth. The seasonal environmental changes and the localization of the ground plots have a great influence on the bivalve population development, namely in survival, growth and reproduction, for example the quality and availably of food are factors of great importance to the bivalve ground plot productivity (Royo, 1986; Abalde et al., 1990). The environmental quality assessment of bivalves' ground plots requires the evaluation of integrated biological and physiological effects. The physiological and biological aspects are regulated by several natural environmental factors like water quality, availability of food, pollution, etc. (Kraeuter and Castagna, 1989). Proper evaluation of the effect of these parameters is essential for the improvement of bivalve production.

The ecological and economic importance of filter feeding shellfish justifies the numerous studies that have been developed to better understand the mechanisms that control the biological and ecological quality of the bivalves. The study of bivalve biological and physiological aspects could help to identify, explain and predict the effects of the natural aquatic environments (Rhodes and Widman, 1984).

Production of *R. decussatus* seed in hatcheries is a relatively new industry for which most methods have been developed using empirical approaches, adapting methods across species and measuring the resulting effects in terms of growth and survival. Applied research on biochemistry and physiology could allow a better understanding of the effect of biotic (microalgal diet, nutritional requirements, etc.) and abiotic (temperature, salinity, etc.) factors.

Due to high social and economic importance of *R. decussatus* it seems essential to optimize the production conditions of this species. Thus, research related with studies on reproduction, nutrition and the support environmental conditions to improve the production technologies of this species are then of utmost importance in order to ensure the economic viability of its culture.

1.2. Ruditapes decussatus: Biology and Ecology *versus* Production Phases

The objective of this item is to present: i) a general overview of *R. decussatus* biology and ecology; ii) the main life cycle stages of this species with in parallel the corresponding production phases.

1.2.1. Taxonomy and distribution

The taxonomic classification according to Howson and Picton (1997) is as follows:

Phylum – Mollusca; **Class** – Bivalvia; **Subclass** – Heterodonta; **Order** – Veneroida; **Superfamaly** - Veneroidea; **Family** - Veneridae; **Genus** – Ruditapes; **Species** – *Ruditapes decussatus*

R. decussatus has a native distribution that extends from Norway to Somalia, along the Iberian Peninsula, and into the Mediterranean Sea (Parache, 1982) (see Figure 1.1). In Portugal, it is present at the “Ria de Aveiro”, “Lagoa de Óbidos”, estuaries of Tejo, Sado and Arade, “Ria de Alvor” and Ria Formosa (Vilela, 1950). It is also present in São Jorge Island, Açores more precisely in “Lagoa da Fajã de Santo Cristo” (Jordaens et al., 2000).

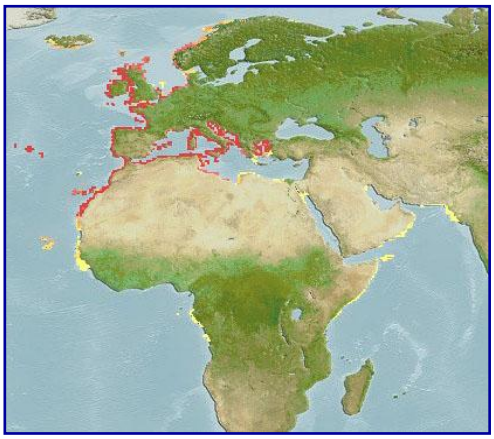

Figure 1.1. Geographical distribution of *Ruditapes decussatus* (Computer Generated Map for *Ruditapes decussatus* (un-reviewed). www.aquamaps.org, version of Aug. 2010).

1.2.2. Biology

1.2.2.1. External anatomy

The shell has fine concentric striae and bolder radiating lines, giving a characteristic squared appearance. The lines of growth are clear. Colour may vary from white to brown depending on the colour of the sediment. Each valve presents three cardinal teeth: the central one in the left valve, and the central and the posterior in the right are bifid. Pallial line and adductor scars are distinct (Poppe and Goto, 1991) (Figure 1.2).

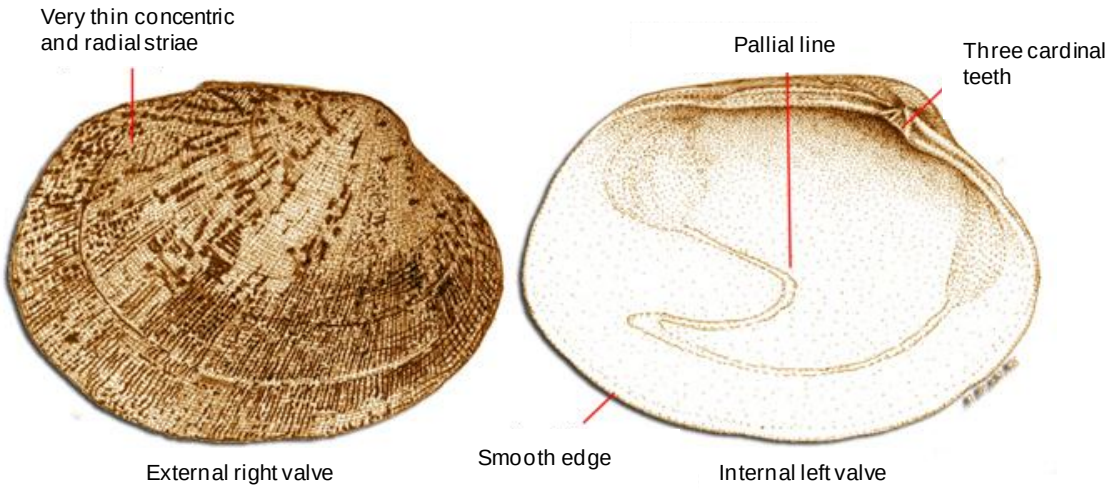

Figure 1.2. *Ruditapes decussatus* external and internal features of the shell valves (www.ictioterm.es).

1.2.2.2. Internal anatomy

In general, the internal anatomy of *R. decussatus* is characterized by the following structures and systems (Cesari and Pellizzato, 1990) (see Figure 1.3):

1. **Mantle** - The soft parts are covered by the mantle, which is composed of two thin sheaths of tissue, thickened at the edges. The main function of the mantle is to secrete the shell. However, this structure also has a sensory function and can initiate closure of the valves in response to unfavourable environmental conditions. It can also control inflow of water into the body chamber and, in addition, has a respiratory function.
2. **Adductor muscle(s)** - The two adductor muscles are located near the anterior and posterior margins of the shell valves. The muscle(s) close the valves and act inopposition to the ligament and resilium, which spring the valves open when the muscles relax.

3. **Siphons** - Its siphons are long and separated in their entire length.
4. **Gills and flaps** - The gills are large leaf-like organs that are used partly for respiration and partly for filtering food from the water. Two pairs of gills are located on each side of the body. At the anterior end, two pairs of flaps, termed labial palps, surround the mouth and direct food into the mouth.
5. **Foot** - At the base of the visceral mass is the foot. It is a well developed organ that is used to burrow into the substrate and anchor the animal in position.
6. **The digestive system** – The digestive system is simple. The large gills filter food from the water and direct it to the labial palps, which surround the mouth. The food is taken into the labial palps to the mouth, which is connected to the stomach for a short esophagus. From the stomach, the food continues to the following digestive diverticulum and intestine. The intestine ends at the anus, which is located in the cloacal chamber, near the adductor muscle.
7. **The circulatory system** – The circulatory system is simple and an open type one, consisting of veins, arteries, heart, pericardium, and tissue, with circulating hemolymph.
8. **The nervous system** – The nervous system is quite simple and essentially consists of three pairs of ganglia (cerebral, pedal and visceral).
9. **Urogenital system** - Sexes are generally separate (dioecious). The gonads occupy a major portion of the visceral mass and are generally only evident during the breeding season. Microscopic examination of the gonad is required to determine the sex of the animal. A small degree of hermaphrodism may occur (Delgado and Pérez-Camacho, 2002). The renal system is difficult to observe.

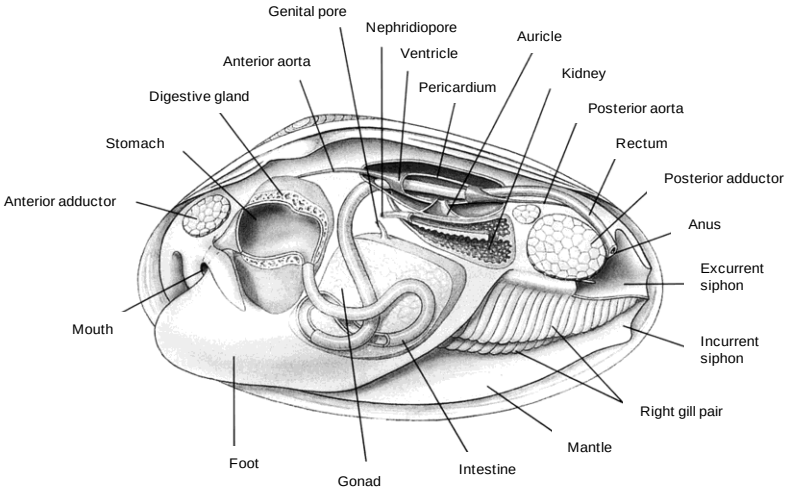

Figure 1.3. Schematic representation of *Ruditapes decussatus* internal anatomy (adapted from Gosling, 2002).

1.2.3. Life cycle versus production phases

The life cycle of *R. decussatus* is the typical of most clam species (Figure 1.4).

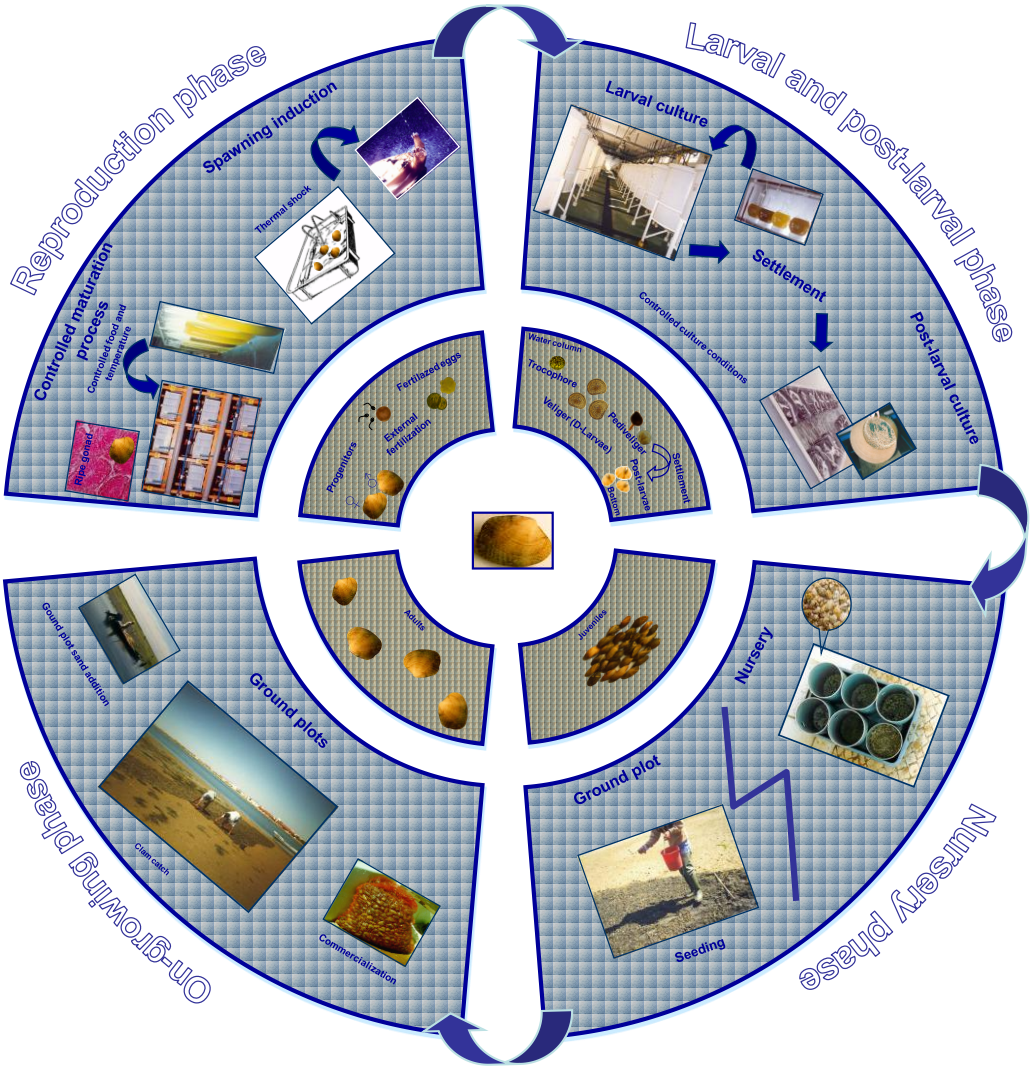

Figure 1.4. Schematic *Ruditapes decussatus* life cycle *versus* production phases.

1.2.3.1. Reproduction

R. decussatus sexual maturity is dependent on size rather than age and geographic distribution (Ojea et al., 2004). The sexes are generally separate and sexual maturity is generally attained when the clams are about 20 mm. The gonad is composed of many-branched, ciliated ducts from which numerous sacs, termed follicles, open. Gametes arise by proliferation of germinal cells that line the follicle wall. The gonad undergoes continuous development until it becomes fully mature but this development has been divided into several stages for convenience, *e.g.* resting, developing, mature, partially spawned and spawned (Delgado and Pérez-Camacho, 2007). When the gonads or gonadal tissue are fully mature they are very evident and form a significant portion of the soft parts of the animal. Gonaducts that will carry the gametes to the body chamber develop, enlarge and are readily observed in the gonad. At this time the animal is frequently referred to as being spwan (Shafee and Daoudi, 1991; Serdar et al., 2010).

In bivalves, the gametogenesis (production of eggs and sperm) depends on the synergetic effects of both internal and external factors, most often temperature and quantity and quality of food are undoubtedly important in initiating this process (*e.g.* Cesari and Pellizzato,1990; Massapina et al., 1999). In generally, *R. decussatus* in the end of winter/beginning of spring, the gonads of the male and female begin to ripening. Once the individual clams are ripe, some stimulus, often a rapid rise in temperature will trigger spawning (Pérez-Camacho et al., 2003). Spawning may be triggered by several environmental factors including temperature, chemical and physical stimuli, water currents or a combination of these and other factors. The period of spawning in natural populations differs with geographic location (Ojea et al., 2004).

In hatchery production, wild breeders of bivalves can be collected at maturity in natural environments during the period preceding their reproductive season, but this period is limited to 2 to 3 months. It is an important aim of hatchery to extend this period of reproductive maturity and thus to produce conditioned hatchery broodstock throughout the year by following controlled procedures. A way to induce sexual maturation in bivalves is the manipulation of the physical and nutritional conditions (Gallager and Mann, 1986; Delgado and Pérez-Camacho, 2005). Adults taken from the natural environment are brought into the hatchery and placed in tanks. The broodstock are kept in the best possible conditions of high water flow, temperature and food availability. Cultured marine algal species are used as the principal food supply during conditioning. *R. decussatus* will require 4 to 8 weeks of conditioning to reach spawning readiness during late winter and early spring (Delgado and Pérez-Camacho, 2005; Joaquim et al., 2008). When the individual are at ripe stage, spawn are induce, most frequently, by thermal stimulation (Joaquim et al., 2008). According to Cesari and Pellizzato (1990), in bivalve, in general, a progressively shorter period of broodstock conditioning will be required as the natural

breeding season approaches. Precise timing depends on the initial condition of the broodstock, stage in gametogenesis when the bivalves begin conditioning and hatchery related factors, the most important of which are temperature, diet and ration.

1.2.3.2. Spawning and fertilization

Natural spawning of *R. decussatus* is confined to a particular time of the year (between May to October) (Banha, 1984). Eggs and sperm are released into the water where fertilization occurs. The presence of sperm in the water will frequently trigger spawning in other animals. Sperm is discharged in a thin, steady stream through the exhalent opening or exhalent siphon. Discharge of eggs is more intermittent and they are emitted in clouds from the exhalent opening or siphon (Cesari and Pellizzato, 1990). Fertilization occurs in water.

In hatchery production, spawning is the procedure by which conditioned bivalves are induced to liberate their mature gametes in response to applied stimuli. In the case of *R. decussatus*, viable embryos cannot be obtained from "stripped" gametes; eggs need to undergo a maturation process during passage down the oviducts before they can be successfully fertilized (Hamida et al., 2004). Mature clams are placed in a spawning tank and spawning is induced by thermal shocks between 5 ºC to 28 ºC, at one hour interval (Joaquim et al., 2009). The number of cool/warm cycles that are required to induce spawning depends on the state of maturity of the gametes and the readiness of the adults to spawn. Generally, if the adults do not respond within a 3 to 4 h period they are returned to the conditioning tanks. Adults may start spawning on either the cool or warm part of the cycle, most commonly the warm. Additional stimuli can be provided in the form of stripped eggs, or sperm from an opened individual. The first adults to spawn are almost always males. As each male and female begin to spawn it is necessary to remove it from the spawning tank and transfer it to an individual spawning beaker with filtered seawater at same temperature (Joaquim et al., 2008). The batches oocytes and sperm are pooled separately and gently washed into a clean glass. Small volumes of the pooled sperm suspension are mixed with eggs during gentle agitation aiming to otain around 10 spermatozoids by oocyte (Cesari and Pellizzato, 1990; Joaquim et al., 2008).

1.2.3.3. Embryonic, larval development and settlement and metamorphosis

R. decussatus fertilized eggs develop into straight-hinge, D" or Prodissoconch I stage, free swimming larvae within 48 h, passing through the multicelled blastula and gastrula stages and a motile trochophore. The subsequent shelled larva is called a veliger larva because of its velum with which it swims and eats. *R. decussatus* veliger stage lasts for about three weeks, during which it grows to approximately 230 µm and the shell becomes umbone or

Prodissoconch II stage (Cesari and Pellizzato, 1990; Joaquim et al., 2008). At this point it becomes a pediveliger and both crawls with its foot and swims with its velum looking for a suitable habitat for adult growth. During metamorphosis a foot develops and gill rudiments become evident and the larvae changes from a swimming, planktonic to a sedentary benthic existence post-larvae (Gosling, 2002).

In hatchery production, *R. decussatus* fertilized eggs are incubated at 20 ºC in conical tanks with filtered and ultraviolet treatment seawater with slight aeration. Embryo stocking densities are of 100,000 per litre. Fully developed D-larvae are recovered 48 h later and tanks containing newly developed D-larvae are drained to a sieve battery, 2 days after fertilization. The larvae retain in a beaker are homogenized and small sub-samples are often taken to be subsequently measured for shell length and evaluate survival. After these operation larvae are transferred to the tank at a larval density of 10,000 larvae per litre (Joaquim et al., 2008). Larvae are now at the stage where they need feeding with unicellular cultured microalgae, prior to this time; energy for respiration and development is derived from reserves laid down during egg development (oogenesis) by the maturing females. The rearing tanks are aerated. During larval culture water is change at two days interval. Bivalve larvae swim freely in the water column for much of the larval phase. As they develop towards the end of the larval phase, feeding activity slows, less food is consumed, and larvae spend an increasing period of time towards the bottom and on the bottom of the tank (Lane et al., 1985; Beaumont and Barnes, 1992; Hiddink et al., 2002). This marks the beginning of metamorphosis. Successful transformation and survival to the juvenile form is dependent on a number of factors, not least of which is the availability of energy reserves accumulated during the larval phase (Loosanoff and Davis, 1963).

1.2.3.4. Post-larvae and juvenile

After the larval stage, *R. decussatus* start their benthonic phase, settling on muddy or sandy sediment, in intertidal areas. The burrowing depth is of approximately 10 to 12 cm, depending on various factors such as consistency of the sediment, population density, the physiological state of individuals, as well as the size of siphons. This species tolerates salinities of 20 to 40 and the optimal temperature for growth is 20-22 °C, the clam stop growing below 12 °C (Vilela, 1950). Growth of juveniles depends greatly of the geographic localization, however a seasonal trend could be describe: growth is generally rapid during spring and summer when food is abundant and water temperatures are warmer and it virtually ceases in winter, resulting in annual growth line in the shell (Matias, 2007). *R. decussatus* can grow up to 7.5 cm in length, however commercial size (3.5 cm) are attain in two years. This species tends to bury itself in sand, muddy gravel or clay and is found on the intertidal and shallow sublittoral (Beninger and Lucas, 1984).

In aquaculture production, this step is divided in two phases: post-growing and on-growing. In post-growing phase, bivalves, from settling size to approximately 2 mm shell-height the postlarvae are reared in flow-through system indoor conditions and fed with cultured microalgae (Loosanoff and Davis, 1963). Thereafter seed is transferred outdoors into the natural environment (temperature and salinity) until it reach saleable size for future stages of farming (Helm et al., 2004). The on-growing phase aims to grow seeds to commercial size as quickly as possible to make the operation economically attractive. *R. decussatus* on-growing phase is based on a series of sequential steps. Firstly, the ground plot is prepared according to the evaluation of the quality of the natural substrate; in fact depending on the evaluation sand and/or gravel are added, in order to increase sediment oxygenation. The seeding density varies, depending on the size of the juveniles and on the characteristics of the area. It usually varies from 300 to 1,500 individuals per m^2. Afterwards it is usual to consider a growth phase, from March to October, and a resting growth phase, from November until February. However, these periods may change depending on the hydrological conditions at the precise location of the ground plot, which determines the duration of air-exposure, water residence-time, currents and nature of the substrate (Matias, 2007). Clams are usually left to grow in the grounds plots for 1.5 to 2 years after transplantation, depending on the initial size of the seed. Ground plots are maintained and cleaned during this on-growing phase. Ground plots are harvested periodically, in a rotation system, in order to have two intense periods of harvest: winter (December and January) and summer (July, August and September). The common technique for harvesting the clams consists on digging and tilling the sediment with a modified knife with a large blade.

1.3. Objectives

The **general purpose** of this study is then to develop a *R. decussatus* production program based on the evaluation of the biological and ecological processes at different culture phases (broodstock conditioning, larval culture and on-growing). This thesis aims to provide new biological and ecological data in order to answer four main questions: (1) How can we **optimise reproductive development** in *R. decussatus* broodstock? (2) What are the mechanisms and major factors involved in *R. decussatus* **embryonic development** and consequent larval viability? (3) How can diet affect *R. decussatus* **larval viability**? (4) How a seasonal and spatial pattern of environmental factors affects the physiological and biochemical processes of *R. decussatus* during the on-growing phase?

1.3.1. Outline of the thesis

Aiming to answer the questions presented above, this thesis was structured in following chapters:

Chapter 2 characterizes the reproductive cycle of the two main populations of *R. decussatus* (Ria Aveiro and Ria Formosa Lagoon) as well as its nutrient storage and exploitation strategy. Inherent physiological variability among bivalve larvae can always be expected in production situations. Moreover, broodstock natural condition is an important factor that contributes to this variability. Understanding the natural reproductive cycle of the different populations is then essential for the establishment of a successful hatchery-based production.

Chapter 3 evaluates the effect of geographic origin, temperature and timing of broodstock collection on conditioning, spawning success and larval viability of *R. decussatus*. Artificial reproduction of bivalves requires the use of animals that have attained an optimal sexual condition, which depends on the synergetic effect of both internal and external factors. Most often temperature and food availability are considered the key external factors. The conditioning required to bring broodstock to the optimum stage of gonadal development is also dependent upon the stage of gonadal development at the beginning of the conditioning and upon the conditioning rate. The aim of this chapter was then to identify the most adequate conditioning temperature for the two geographically (Ria Formosa and Rias Galegas) distinct populations in order to create ‘‘optimal’’ artificial spawning and larval rearing programs.

Chapter 4 assesses the effects of different diets and temperatures on broodstock performance of *R. decussatus*. Food and temperature are the main factors that regulate the timing and rate of energy storage and reproduction in bivalves. The effect of these variables is complex and depends specifically on the acquisition and expenditure of energy. In this chapter we evaluated the effects of different diets and temperatures on reproductive output of *R. decussatus* and express the evolution of the different lipid classes during sexual maturation, aiming to find a broodstock conditioning diet and temperature that maximizes fecundity and egg quality and that could be suitable for commercial hatcheries.

Chapter 5 describes the biochemical compounds dynamics during larval development of *R. decussatus* and the effect of monospecific diets and starvation on these dynamics. Successful bivalve larval growth and development depends on the energy reserves stored during endotrophic and exotrophic phase. In this study we evaluated the duration of the transition period from the endotrophic to the exotrophic phase and analyzed the biochemical changes in starved larvae to obtain information on the nutritional requirements and consequently a better understanding of normal (*i.e.* fed) larval development. The results obtained could provide useful information for the artificial production of *R. decussatus*.

Chapter 6 evaluates the influence of different mono and bispecific diets on *R. decussatus* larval rearing performance. Optimizing larval survival and nutrition are crucial aspects of overall hatchery production. Microalgae are the primary food source used in aquaculture as live feeds for all growth stage of bivalves. Meeting the specific diet requirements of clam larvae will depend not only on the concentration, but also composition of feed. The aim of this study was to determine the effect of different diets (monospecific and bispecific in different proportions) on the survival, growth, settlement and biochemical composition of hatchery reared larvae. The information on the nutritional requirements of hatchery reared *R. decussatus* larvae obtained will be useful for the establishment of feeding practices that maximise larvae yield and minimise cost.

In **Chapter 7** we evaluated the effect environmental factors on the culture performance *R. decussatus*. Highly productive areas such as estuaries and coastal lagoons are at risk due to increasing stress from anthropogenic activities such as urbanization, industrialization, intensive agriculture and mass tourism. In this context, there is an on-growing interest in the influence of environmental factors on biota biology and physiology, namely organisms' survival and growth, and changes in biochemical composition, especially in energetic reserves cycles (glycogen and total lipids), as a reflection of reproduction. In this study we compared biological (growth, allometry, survival and condition index) and physiological (biochemical composition) parameters of *R. decussatus* juveniles originating from two clams' ground plots located in different sites of the Ria Formosa Lagoon, and subjected to opposite environmental conditions. The results obtained in this study could also be useful to design a geographic information system with the better and worst areas for clam production, contributing to provide adequate management strategies.

Based on the most pertinent results obtained for clam production in the different chapters of this thesis, a set of production and management strategies are proposed in **Chapter 8** as a basis for promoting the sustainable *R. decussatus* production.

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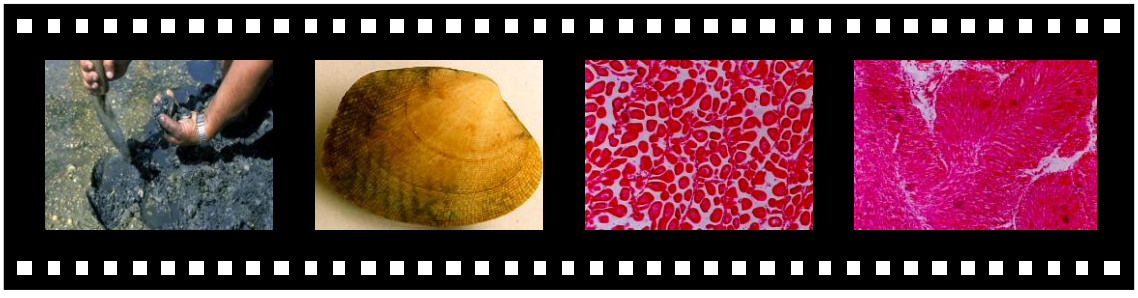
Chapter 2

The reproductive cycle of the European clam *Ruditapes decussatus* (Linnaeus, 1758) in two Portuguese populations: implications for management and aquaculture programs

Matias D., Joaquim S., Matias A.M., Moura P., Teixeira de Sousa J., Sobral P., Leitão A. The reproductive cycle of the European clam *Ruditapes decussatus* (Linnaues, 1758) in two Portuguese populations: implications for management and aquaculture programs. Aquaculture (submitted).



Abstract

Ruditapes decussatus is widely distributed along the coastal and estuarine areas of Europe and Northern Africa and represents an important resource income due to its high commercial value. However in Portugal, during the last two decades, the European clam production has suffered an important decrease due to several constrains, namely recruitment failures and excessive pressure on the capture of juvenile on natural banks and severe clam mortalities. To overcome this situation, artificial spawning and larval rearing programs could provide an alternative source of spat. The detailed knowledge of the reproductive cycle is crucial to establish and improve rearing programs for *R. decussatus*. The reproductive cycle, as well as its nutrient storage and utilization, of two populations of *R. decussatus* from the main production areas of this species: Ria de Aveiro and Ria Formosa Lagoon (Portugal) were studied here over a 24 months period (May 2010 – April 2012). The reproductive cycle of both populations of *R. decussatus* followed an annual cyclicality that comprised a ripe stage in spring followed by a spawning period that began in late spring and extended throughout summer until early autumn. This extended and continuous spawning period may be an advantageous strategy for this species by ensuring a continuous supply of gametes. Moreover, *R. decussatus* can adopt different reproductive strategies depending on the geographical origin. The results of both cycle of nutrients stored and nutrients utilization showed that clams of both populations present a high reproductive effort that almost depletes its energy reserves. Nevertheless, while Ria de Aveiro population retrieves them immediately after spawning, the same is not verified in clams from Ria Formosa Lagoon with their consequent debilitation. Also, based on the glycogen pattern it was possible to infer that the Ria Aveiro population is an opportunistic one, while the Ria Formosa Lagoon population exhibited an intermediate strategy. However, both populations could be considered as viable broodstock for intensive hatchery production of juveniles and the observed extended spawning periods has interesting implications for the implementation of profitable aquaculture. Moreover, this species presented a great capacity for gonadal regeneration, which coupled with its high gonadal development rate would provide larvae during most of the year without extensive and expensive broodstock conditioning.

Keywords: European clam; *Ruditapes decussatus*; Reproductive cycle; Portuguese populations; Biochemical composition; Seasonal variations.

2.1. Introduction

The European clam *Ruditapes decussatus* is widely distributed along the coastal and estuarine areas of Europe and Northern Africa and represents an important resource income due to its high commercial value (Matias et al., 2011). *R. decussatus* is extensively produced and harvested in Portugal, where clam farming represents an important economical sector. This species is central to aquaculture's revenue, indeed in 2009, the national annual production reported reached 2 metric tons (representing 27 % of the total seafood cultured in Portugal) (DGPA, 2011). The main production areas of this species are the Ria de Aveiro (40º42′N; 08º40′W) and the Ria Formosa Lagoon (37º01′N; 07º49′W). The culture of *R. decussatus* in Ria Formosa Lagoon represents 90 % of the national production and it is central to the socioeconomic framework. However, during the last two decades, the European clam production has suffered an important decrease due to several constrains, namely recruitment failures and excessive pressure on the capture of juvenile on natural banks and severe clam mortalities.

To address this situation, artificial spawning and larval rearing programs could provide an alternative source of spat.

To be able to establish and improve rearing programs for *R. decussatus*, a detailed knowledge of the species reproductive cycle and spawning periods is crucial. Effectively, the differences in gonadal cycles and conditioning optima in different populations have to be considered in hatchery operations (Lannan et al., 1980; Devauchelle and Mingant, 1991). There is also evidence that responses also vary between different geographical populations of the same species, as has been found for *Mytilus galloprovincialis* (Iglesias et al., 1996) and *Argopecten purpuratus* (Avendaño and Le Pennec, 1997). In the case of the European clam, in natural conditions, it has been reported that the ecotype decussatus living in different areas, even at the same latitude, could strongly differ in terms of their fecundity levels and biochemical compositions (Shaffee and Daoudi 1991; Trigui-El-Menif et al. 1995).

A relationship between the reproductive cycle and energy storage and utilization cycles has also already been reported by several authors for a wide variety of bivalves (*e.g.* Barber and Blake, 1981; Fernández-Castro and Vido-de-Mattio, 1987; Massapina et al., 1999; Pérez-Camacho et al., 2003; Ojea et al., 2004; Joaquim et al., 2011). The energy storage and utilization cycles translate into a seasonal pattern of biochemical composition that can vary according to species and geographical origin (Albentosa et al., 2007; Matias et al., 2009). Energy reserves are of considerable importance in reproduction and seasonal energy storage and utilization in bivalves are closely correlated to environmental conditions and the annual gametogenic cycles (*e.g.* Holland, 1978; Delgado et al., 2004; Ojea et al., 2004; Tlili et al., 2012). Food and temperature are the main factors that regulate the timing and rate of energy

storage in bivalves (Joaquim et al., 2011). The effect of these variables is complex and depends specifically on acquisition and expenditure of energy (Pérez-Camacho et al., 2003). The most common model consists of an accumulation of energy during the periods where food is abundant. This energy is then used for the gametogenic synthesis and latter released during the spawning process (Albentosa et al., 2007). Proteins are mainly used in structural functions and represent an energy reserve in adult bivalves, particularly during gametogenesis and in situations of low glycogen levels, or severe energy equilibrium (Beninger and Lucas, 1984). Carbohydrates are assumed to constitute the most important bio-energy reserve in bivalve molluscs and, because of their hydro-solubility, are available for immediate use; being glycogen the main component for supplying energy demands (Fernández-Castro and Vito-de-Mattio, 1987) and reproductive cycle (*e.g.* Newell and Bayne 1980; Pazos et al., 2005). Lipids, due to their large calorific contributions per structural unit, account for a greater proportion of the energy reserves in bivalves than carbohydrates or proteins (Ojea et al., 2004). They play an important role in the gamete formation and are the main reserve of oocytes and bivalve larvae (Matias et al., 2009; 2011).

Although previous works have studied the natural reproduction of *R. decussatus* and its biochemical composition (*e.g.* Benninger and Lucas, 1984; Ojea et al., 2004; Serdar and Lök, 2009), in Portuguese populations of this species only the gametogenic cycle has been determined in former studies of Vilela (1950) and Pacheco et al. (1989). Therefore, the present study aims to characterize the reproductive cycle of two populations of *R. decussatus* from the main production areas of this species: Ria de Aveiro and Ria Formosa Lagoon, and also including patterns of nutrient storage and utilization. This information would be essential for the establishment of a successful hatchery-based production.

2.2. Materials and Methods

2.2.1. Sample collection

Generally, samples of *R. decussatus* were hand-collected, monthly, from the two locations in Portugal: Ria de Aveiro and Ria Formosa Lagoon during 24 months (May 2010 – April 2012). Both areas are shallow water mesotidal lagoons with semidiurnal tidal regimes that constitute the major hydrodynamic forces (Nobre et al. 2005; Dias et al. 2000) (Figure 2.1). These lagoons, that distance 500 km from each other, have several channels and a large intertidal area covered by sand, muddy sand-flats, and salt marshes (Falcão and Vale, 1990; Picado et al., 2009). Ria Formosa Lagoon has an extension of 55 km and a maximum width of 6 km (Newton and Mudge, 2003). The lagoon is separated from the Atlantic Ocean by several barrier islands and two peninsulas. The tidal range varies from 1.35 m in neap tides to 3 m in spring tides, and the coefficient of renovation of the lagoon is 3.2 for in spring tide and 1.0 in a neap tide. The freshwater inputs are almost negligible and salinity remains close to 36 all year

long (Falcão and Vale, 1990; Águas, 1986). The Ria de Aveiro is 45 km long and 10 km wide, being connected to the Atlantic Ocean by only a narrow channel (Picado et al., 2009), and the tidal amplitude is 0.6 m in neap tides and 3.2 m in spring tides (Dias et al., 2000). This lagoon has an important freshwater input coming from the Vouga and the Antuã rivers (Moreira et al., 1993; Dias et al., 2000) and salinity ranged between 31 and 36. These two ecosystems are currently used for clam production and fish aquaculture ponds.

Monthly data on sea surface temperature (SST) during the study period were derived from satellite remote sensing data, collected from the Giovanni online data system (MODIS-Aqua 4 km, monthly processed data, available at http://disc.sci.gsfc.nasa.gov/giovanni/overview/index.html, developed and maintained by the NASA Goddard Environmental Sciences Data and Information Services Center - GESDISC (Acker and Leptoukh, 2007).

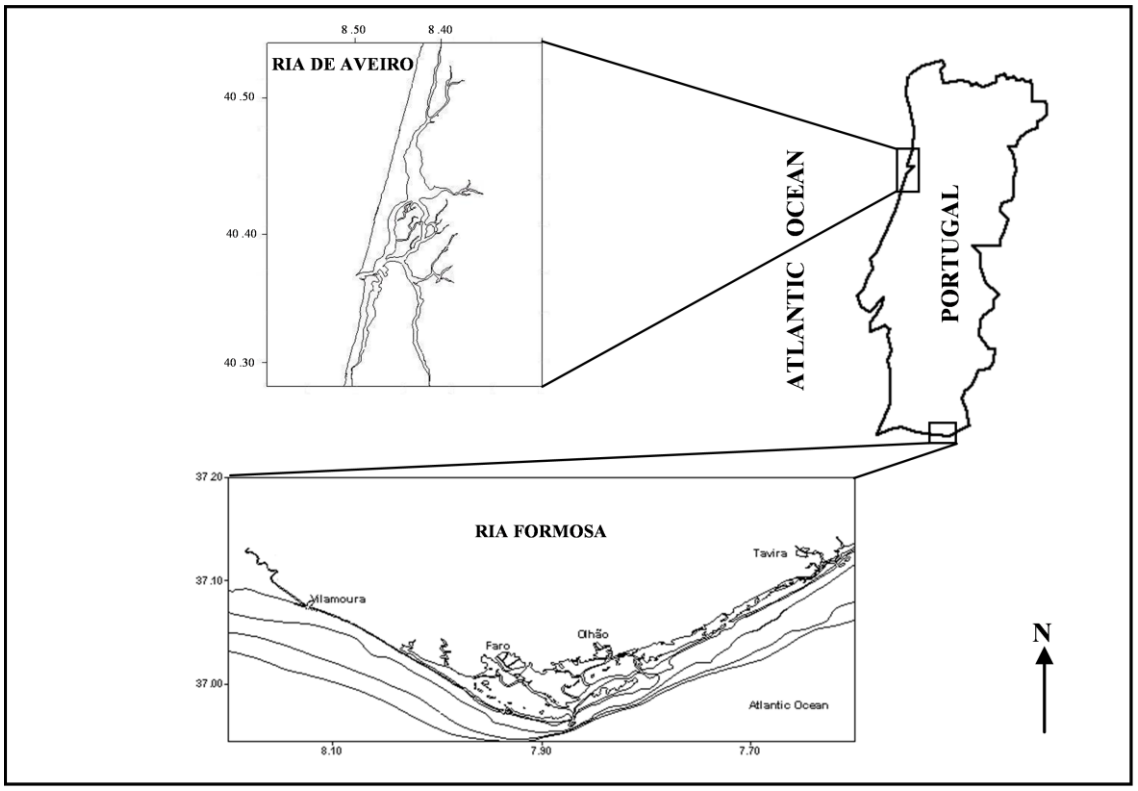


Figure 2.1. Collection locations of the two *Ruditapes decussatus* populations.

2.2.2. Laboratory analysis

In the laboratory, clams were placed in 0.45 µm-filtered seawater at 20 °C for 24 h to purge their stomachs before histological, condition index and biochemical analyses.

2.2.2.1. Histology

Ten individuals of each sex from each monthly sample and each population were examined histologically to determine the gametogenic stages in both sexes. The visceral mass was separated from siphons and gills and fixed in Davison solution for 48 h, then transferred to 70 % ethyl alcohol (ETOH) for storage. Tissues from these samples were dehydrated with serial dilutions of alcohol and embedded in paraffin. Thick sections (6–8 µm) were cut on a microtome and stained with haematoxylin and eosin. The histologically prepared slides were examined using a microscope at 40× magnification and each specimen was assigned to a stage which represented the gonadal state. Clam reproductive maturity was categorized into six stages using a scale development based on Delgado and Pérez-Camacho (2005) (Table 2.1 and Figures 2.2 and 2.3). When more than one developmental stage occurred simultaneously within a single individual, the assignment of a stage criteria decision was based upon the condition of the majority of the section.

A mean gonadal index (GI) was calculated using the method proposed by Seed (1976): GI = [($\sum$ ind. each stage X stage ranking) / total ind. each month].

For each of the stages a numerical ranking was assigned as follows: Period of sexual rest (0); initiation of gametogenesis (3); advanced gametogenesis (4); ripe (5); partially spawned (2); spent (1). The GI ranged from 0 (all individuals in the sample are in rest stage) to 5 (all individuals are in ripe stage).

Table 2.1. Reproductive scale for *Ruditapes decussatus* development based on Delgado and Pérez-Camacho (2005).

Stage	Histologic description
Period of sexual rest (phase I)	Gonadal follicles are absent and connective and muscular tissue occupies the entire zone from the digestive gland to foot. There is no evidence of gonadal development and sex determination is not possible.
Initiation of gametogenesis (phase II)	Follicles and gonadal acini begin to appear in females and males. They increase in size, and appear covered with oocytes in the growth phase in the females and with immature gametes They increase in size, and appear covered with oocytes in the (spermatogonia and spermatocytes) in the males.
Advanced gametogenesis (phase III)	The follicles occupy a large part of the visceral mass. The presence of muscular and connective tissue is reduced. At the end of this stage, characterised by intense cellular growth in females, the oocyte protrudes from the centre of the lumen, remaining attached to the wall via the peduncle. The abundance of free oocytes equals those attached to the wall of the follicle. In males, majority of the acini were full of spermatids and spermatozoids.
Ripe (phase IV)	Corresponding to the maturity of the majority of gametes. In the mature oocytes the rupture of the peduncle occurs, and the oocytes consequently occupy the follicular interior. In males, the gonadal acini mainly contain spermatozoids.
Partially spawned (phase V)	The gametes are discharged. Depending on the degree of spawning the follicles are more or less empty. The follicle walls are broken. There are many empty spaces between and within the follicles. In males, lines of spermatogonias were found in the follicles and in females, ovogonias attached to follicle walls were observed.
Spent (phase VI)	Abundant interfollicular connective tissue. Occasional residual sperm or oocytes resent.

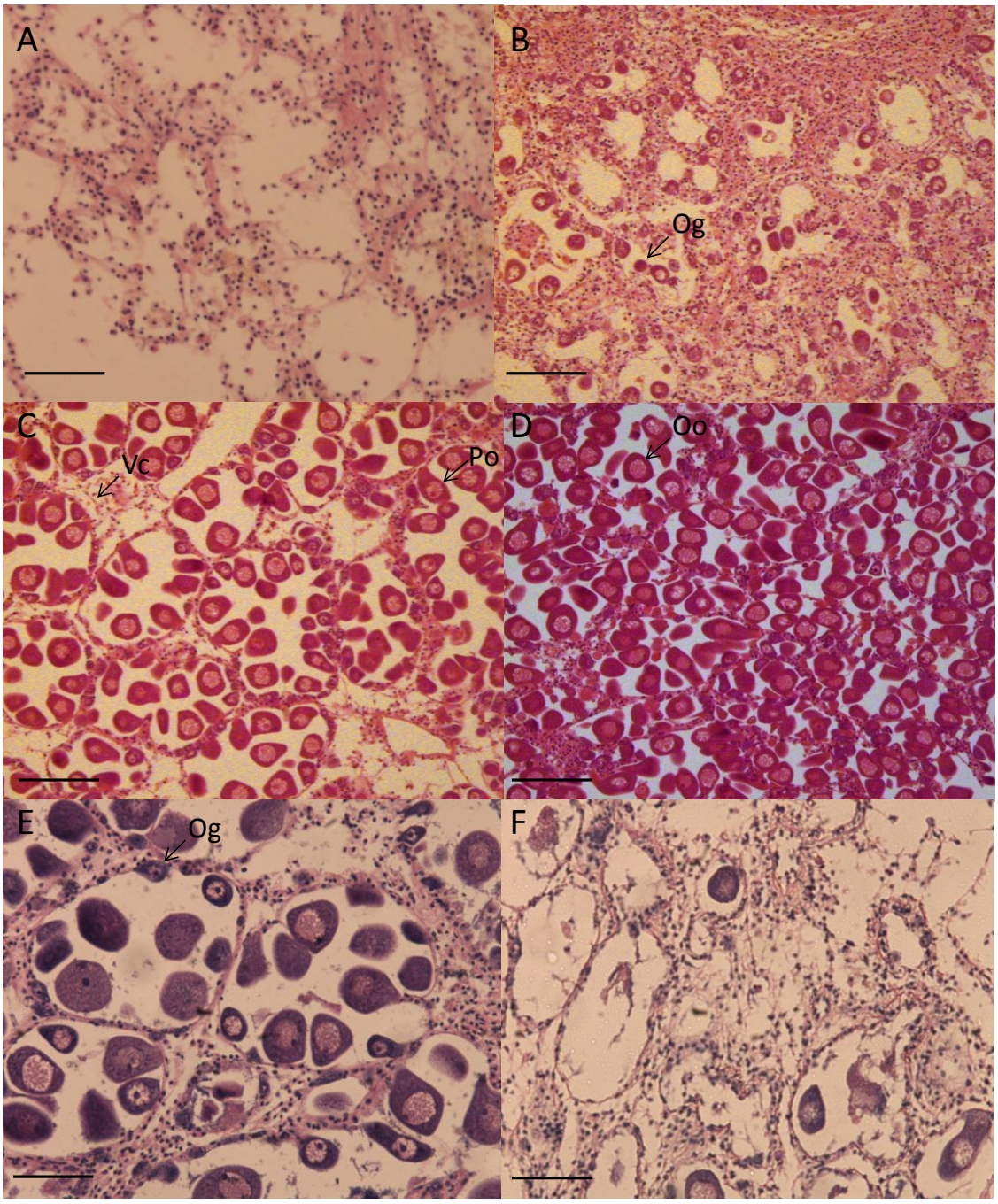


Figure 2.2. Photomicrographs showing stages in the development of *Ruditapes decussatus* female gonad. A. Sexual rest. B. Initiation of gametogenesis; Og – Ovogonia. C. Advanced gametogenesis; Po - Pedunculated oocyte; Vc - Vesicular cell. D. Ripe; Oo – Oocytes. E. Partially spawned; O - Ovogonia. F. Spent. Scale bar: 200 µm in A, B, C and D; 100 µm in E and F.

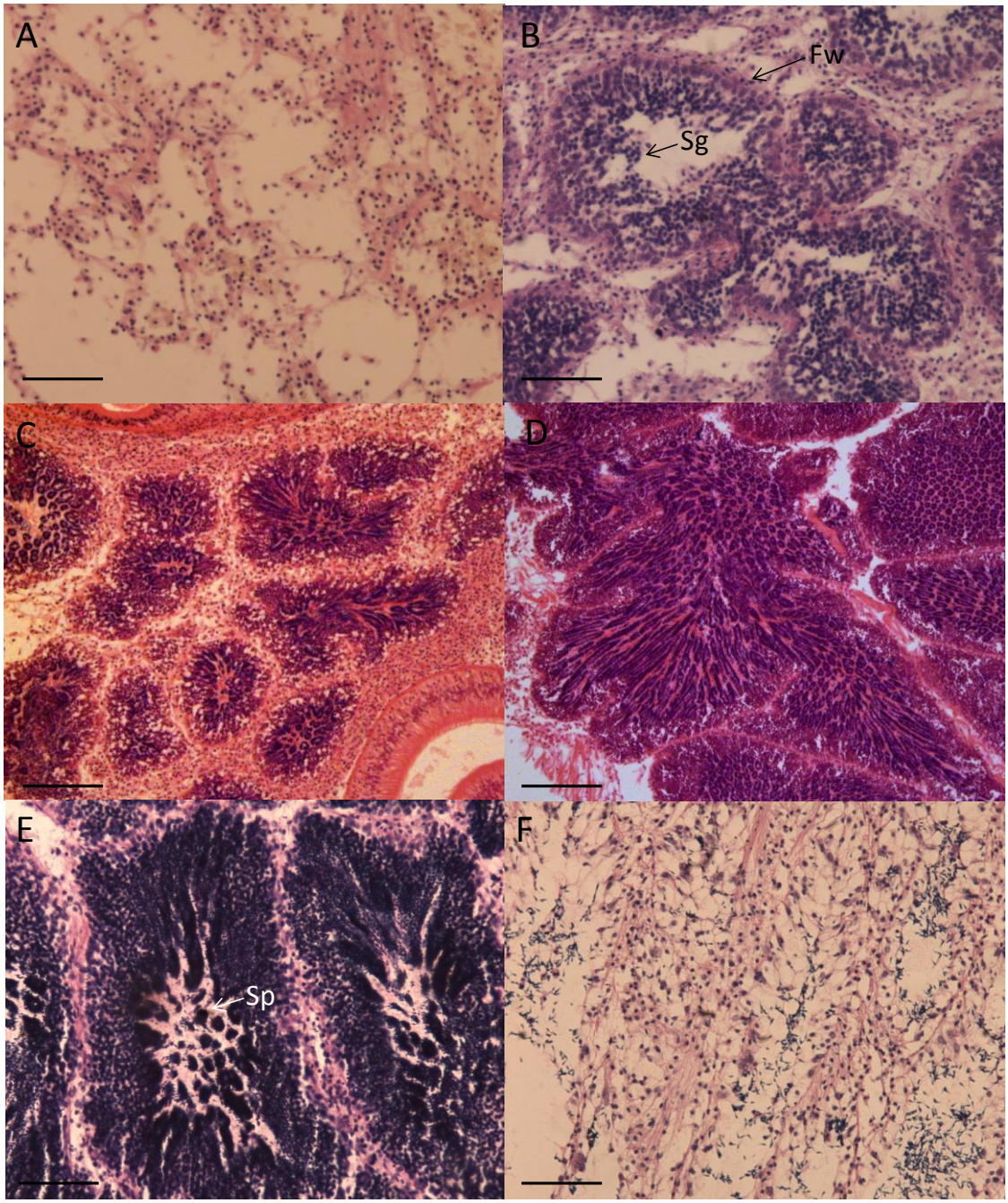


Figure 2.3. Photomicrographs showing stages in the development of *Ruditapes decussatus* male gonad. A. Sexual rest. B. Initiation of gametogenesis; Sg – Spermatogonia; Fw - Follicule wall. C. Advanced gametogenesis. D. Ripe. E. Partially spawned; Sp - Spermatozoa. F. Spent. Scale bar: 200 µm in C; 100 µm in A, B, D, E and F.

2.2.2.2. Condition index

The dry meat and shell weight of 10 clams, from each monthly sample and from each population, were determined after oven drying at 80 °C for 24 h. Meat samples were then ashed at 450 °C in a muffle furnace, ash weight determined, and organic matter weight calculated as the ash free dry meat weight (AFDW). The condition index (CI) was calculated according to Walne and Mann (1975): [ash free dry weight (AFDW) of meat (g)/dry shell weight (g)]*100.

2.2.2.3. Biochemical composition

The meat of ten clams from each monthly sample of the two populations was frozen and stored at −20 °C for biochemical analyses. For each specimen, protein was determined using the modified Lowry method (Shakir et al., 1994), glycogen content was determined from dried (80 °C for 24 h) homogenate using the anthrone reagent (Viles and Silverman, 1949) and total lipids were extracted from fresh homogenized material in chloroform/methanol (Folch et al., 1957) and estimated spectrophotometrically after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966). Duplicate determinations were performed in all cases and values are expressed as a percentage of AFDW. Caloric content of proteins, lipids and carbohydrates in tissues was calculated using the factors 17.9 KJ g^{-1} (Beukema and De Bruin, 1979), 33 KJ g^{-1} (Beninger and Lucas, 1984) and 17.2 KJ g^{-1} (Paine, 1971), respectively.

2.2.3. Statistical treatment of data

Seasonal variations in condition index, biochemical composition and histological parameters were analyzed by one-way ANOVA or Kruskal–Wallis ANOVA on ranks whenever the assumptions of analysis of variance (ANOVA) failed. Percentage data were arcsine transformed to normalize variance (Sokal and Rohlf, 1981). Multiple pairwise comparisons were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test in order to detect significant differences between monthly consecutive samples. The Pearson correlation coefficient was used to determine the degree of association between parameters. Results were considered significant at $P<0.05$. The statistical analyses were performed using the SIGMASTAT 3.11 statistical package.

2.3. Results

2.3.1. Temperature

The evolution of the monthly SST during the experimental period in Ria de Aveiro and Ria Formosa Lagoon is presented in Figure 2.4. Ria de Aveiro presented lower temperature values than Ria Formosa Lagoon (around less 2.95±1.31ºC). A seasonal cycle in SST was observed in the two geographical locations studied, and the monthly means ranged between 19.49 ºC in September 2010 and 12.99 ºC in February 2012 for Ria de Aveiro and 24.01 ºC in August 2010 and 15.03 ºC in February 2012 in Ria Formosa Lagoon.

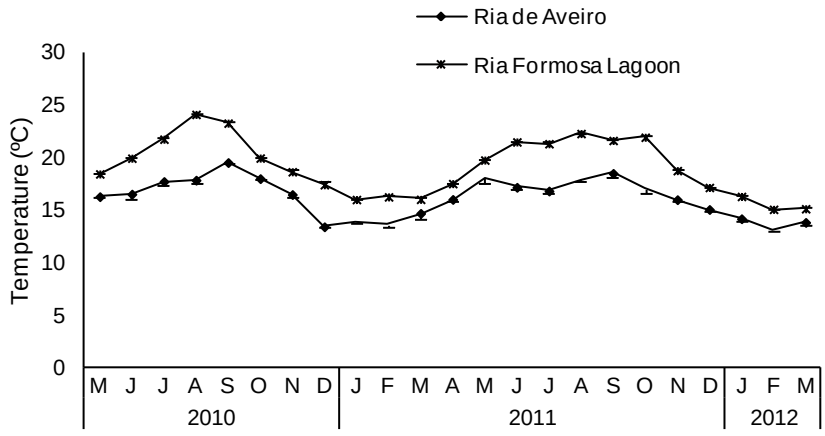


Figure 2.4. Monthly values (mean±SD) of sea surface temperature (SST) in Ria de Aveiro and Ria Formosa Lagoon from May 2010 to April 2012.

2.3.2. Gametogenic cycle

The sexes were clearly separated and no hermaphrodites were found. Both sexes showed synchronism in gonadal development. The reproductive cycle of *R. decussatus* was characterized by a seasonal pattern in both populations (Figure 2.5), however, no significant correlations were found between SST and GI (Figure 2.6) in both populations (Table 2.2).

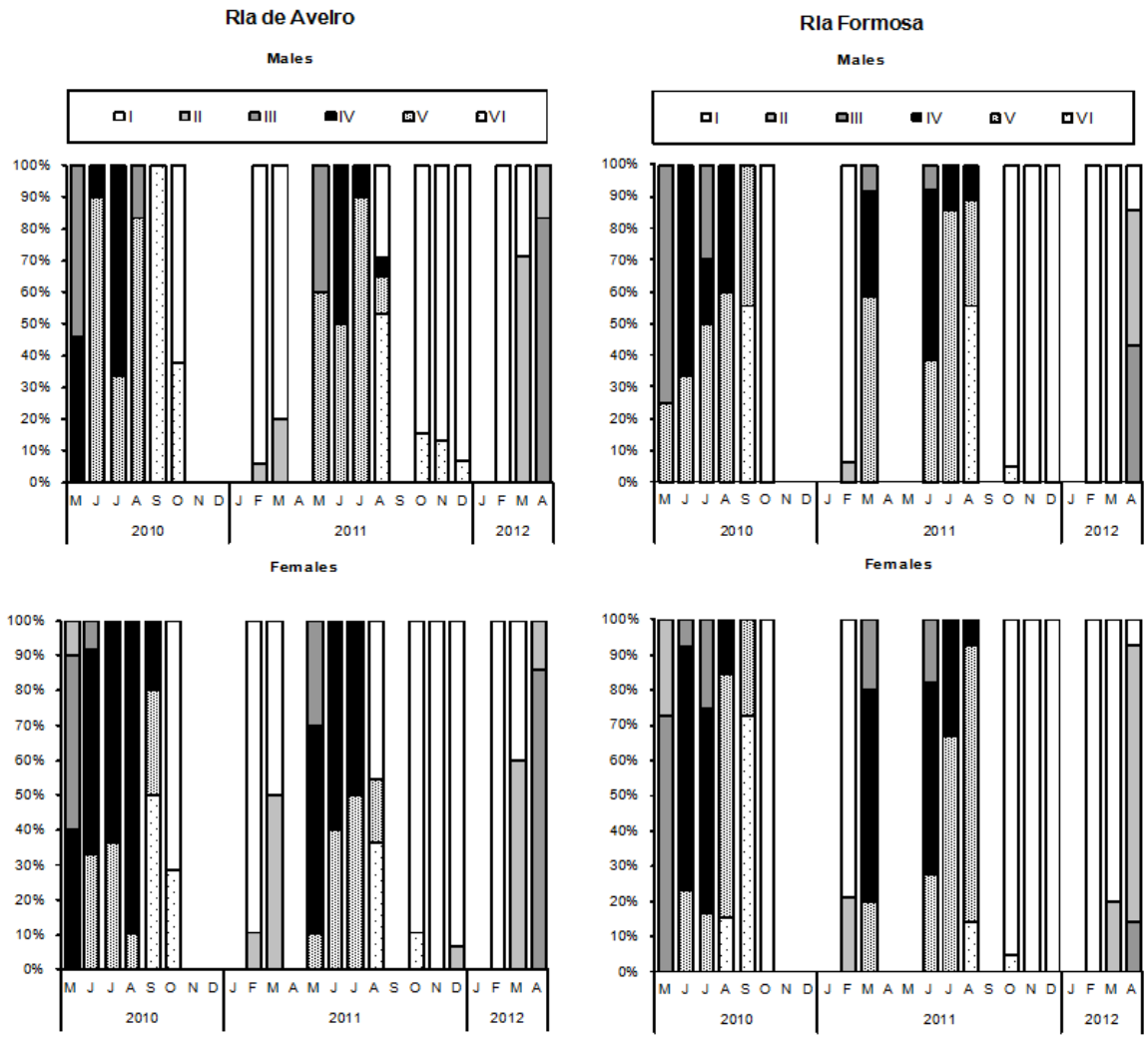


Figure 2.5. Monthly variations in gonadal development of *Ruditapes decussatus* populations from Ria de Aveiro and Ria Formosa Lagoon, during May 2010 to April 2012. Males (top) and Females (bottom).

The onset of the gametogenic cycle occurred in February in 2011 for males and females of both populations, in March in 2012 for males and famales of Ria de Aveiro and in March and April in 2012 for males and females of Ria Formosa Lagoon population, respectively, which coincided with the increase of SST. The development of gametes intensified quickly during the following month. The two populations reached its peak of reproductive effort between May and June, represented by the highest values of GI (Ria de Aveiro: females=4.4 in May and males=3.5 in June; Ria Formosa Lagoon: females=4.2 in May and males=3 in June). Spawning began in late spring for both populations; in 2010, generally in June (except for males of Ria Formosa Lagoon population that began in May) and in March and May 2011 for Ria Formosa Lagoon and Ria Aveiro populations, respectively. Spawning of *R. decussatus* intensified during summer as SST increased, and continued until early autumn in both populations. Nevertheless, during this period and in spite of the seasonal pattern, *R.*

decussatus did not show a continuous gonad development, in which after spawning clams should progress to an inactive stage. Indeed, in the microscopic examinations of the gonadal tissues, all clams showed simultaneous spawning and recovery of the gonad. So, we considered this stage of the reproductive cycle of *R. decussatus* as partially-spawned (stage V – Table 2.1).This phenomenon occurred in all studied years and for both males and females of the two populations. In October the majority of clams had already spawned and were inactive and remained in this stage during approximately six months (which coincided with the decrease of SST) until the next onset of gametogenesis. The gonadal index followed the same pattern as the gonadal development; no significant differences in GI were found between populations or between sexes (ANOVA, P>0.05).

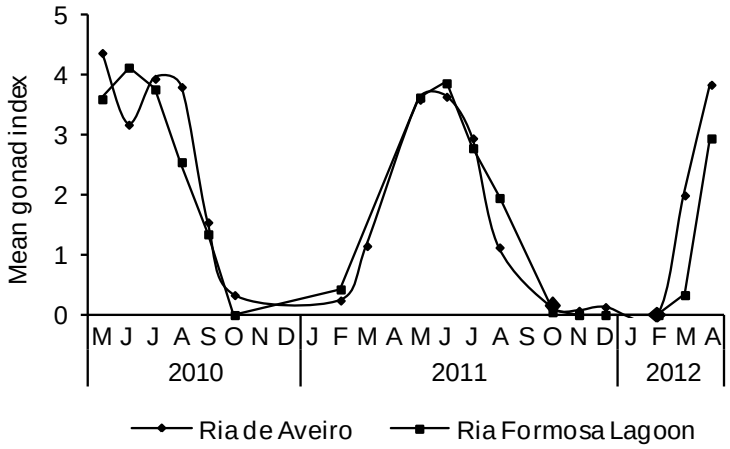


Figure 2.6. Monthly variations in gonad index (GI) (mean, *n*=20) of *Ruditapes decussatus* populations from Ria de Aveiro and Ria Formosa Lagoon, during May 2010 to April 2012.

Table 2.2. Results of Pearson correlation between studied parameters (*r*, correlation coefficient, *P*, *P* value, n.c., no correlation was found).

	Ria de Aveiro						Ria Formosa Lagoon					
	Gonadal index (GI)	Condition index (CI)	Proteins	Total lipids	Glycogen	Total energy	Gonadal index (GI)	Condition index (CI)	Proteins	Total lipids	Glycogen	Total energy
Temperature (SST)	n.c.	*r*= 0.55 *P*< 0.001	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Gonadal index (GI)		n.c.	n.c.	n.c.	n.c.	n.c.		*r*= - 0.87 *P*< 0.001	n.c.	n.c.	*r*= 0.60 *P*= 0.01	n.c.
Condition index (CI)			n.c.	n.c.	n.c.	n.c.			n.c.	n.c.	*r*= 0.78 *P*< 0.001	n.c.
Proteins				*r*= -0.52 *P*< 0.01	n.c.	*r*= 0.87 *P*< 0.001				n.c.	n.c.	*r*= 0.91 *P*< 0.001
Total lipids					n.c.	n.c.					n.c.	n.c.
Glycogen						n.c.						n.c.

2.3.3. Condition index

Condition index exhibited statistically significant differences between populations (K–W., H=7.68, df=1, P=0.006), especially when clams were in the inactive stage, between September and October 2010 and August and November 2011. These differences between populations were also relevant between May and July 2010 (ANOVA, F=26.61, df=7, P<0.001). Significant differences were found between 2010 and 2011 for both populations (Ria de Aveiro: ANOVA, F=12.96, df=1, P<0.001); Ria Formosa Lagoon: K-W., H=13.02, df=1, P<0.001). In Ria de Aveiro population, CI was positively correlated with SST (Pearson, r=0.74, P<0.001) (Table 2.2), however, no correlation was observed between these two parameters in Ria Formosa Lagoon. In 2010, the CI of the Ria de Aveiro population generally trended upwards until September following SST increase (with an exception in July, with a GI contribution), when the highest value (11.96±1.38) of the sampling period was registered (Figure 2.7), coinciding with the end of the reproductive cycle of the species. In the following month the CI decreased coinciding with the end of spawning. However, no relationship was observed between GI and CI for the Ria de Aveiro population (Table 2.2). In the Ria Formosa Lagoon population the CI in 2010 remained high and relatively stable in the first three months of sampling when the majority of clams were in late activity and ripe stage, decreasing sharply from July with the progress of spawning and consequent rest period. The lowest CI value (4.00±0.80) of the sampling period was registered in October 2010 in the Ria Formosa population.

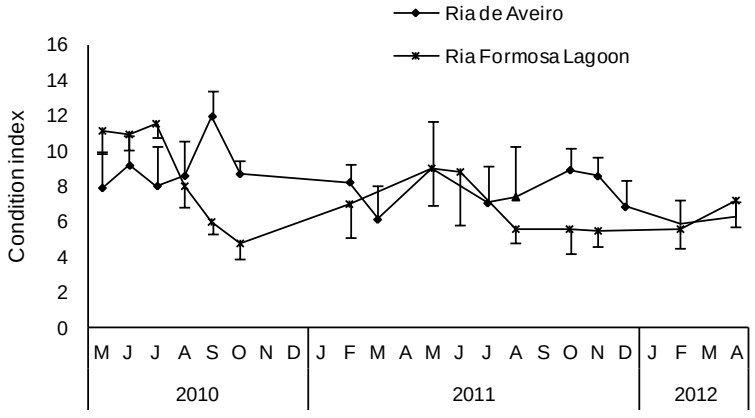


Figure 2.7. Condition index (mean±SD, n=10) of *Ruditapes decussatus* populations from Ria de Aveiro and Ria Formosa Lagoon, during May 2010 to April 2012. (*statistically significant differences, P<0.05 found between populations).

In 2011, Ria de Aveiro population showed two peaks in the CI, the first one in May and another one in October. In contrast, Ria Formosa population only showed one peak in May/June which correspond to the maximum GI value of this year achieved with the contribution of the ripe stage of the most clams. The decline of Ria Formosa Lagoon population CI coincided

with the rest period of clams. The CI of the Ria Formosa Lagoon population was positively correlated with the GI (Pearson, r=0.87, P<0.001) (Table 2.2). Condition index of both populations increased slightly from February to April 2012, following the trend of 2011.

2.3.4. Biochemical composition

Proteins were the predominant dry tissue constituent of the clams followed by total lipids and glycogen (Table 2.3). The highest protein content values were recorded in May 2010 (531.7±180.0 µg mg^{-1} AFDW) and October 2010 (520.8±123.5 µg mg^{-1} AFDW) and the lowest in September 2010 (128.3±32.6 µg mg^{-1} AFDW) and February 2011 (142.2±23.6 µg mg^{-1} AFDW) for Ria de Aveiro and Ria Formosa Lagoon populations, respectively. Significant differences were found between populations (ANOVA, F=4.04, df=1, P=0.045), especially in July, August and September 2010 and February 2011. Also significant differences were observed within populations between years: in Ria de Aveiro clams in May, June and July (K-W., H= 47.78, df=1, P<0.001) and in May and August in Ria Formosa Lagoon population (K-W., H=4.99, df=1, P=0.025). Glycogen content between clams of the two populations showed significant differences (ANOVA, F=58.89, df=1, P<0.001), especially when clams were inactive in terms of reproduction (Period of sexual rest). In this period, the reserves of glycogen were considerably higher in the Ria de Aveiro clams then in the ones from Ria Formosa Lagoon. This content generally decreased in both populations until August 2010. In September 2010, the highest (53.7±22.9 µg mg^{-1} AFDW) and the lowest (7.2±2.4 µg mg^{-1} AFDW) values were recorded for Ria de Aveiro and Ria Formosa Lagoon populations, respectively. After that, the glycogen contents showed opposing trends until February 2011, when the values of the two populations approached. In 2011, glycogen generally followed a similar trend as in 2010, in Ria de Aveiro population (ANOVA, P>0.05) however in the Ria Formosa Lagoon the months of June and July 2011 were significant different from 2010 (ANOVA, F=9.99, df=1, P=0.002). The lowest glycogen values of clams from Ria de Aveiro (9.1±5.8 µg mg^{-1} AFDW) were observed in June 2011. Glycogen was positively correlated with CI (Pearson, r=0.79, P<0.001) and GI (Pearson, r=0.60, P=0.01) in Ria Formosa Lagoon population (Table 2.2). The lowest (Ria de Aveiro: 35.0±9.8 µg mg^{-1} AFDW; Ria Formosa Lagoon: 27.2±7.3 µg mg^{-1} AFDW) and the highest (Ria de Aveiro: 118.1±20.5 µg mg^{-1} AFDW; Ria Formosa Lagoon: 112.1±15.1 µg mg^{-1} AFDW) total lipid values were reached in October 2010 and June 2010 and April 2012 and July 2011, respectively. No significant differences were found between populations for total lipids (ANOVA, P>0.05), however this content had a significantly different trend in 2010 *versus* 2011 in both population [Ria de Aveiro in May, June, July, and October (ANOVA, F=186.15, df=1, P<0.001) and Ria Formosa Lagoon in May, June, July, August and October (ANOVA, F=312.18, df=1, P<0.001)]. Total lipids of clams from Ria de Aveiro population were negatively correlated with proteins (Pearson, r=-0.52, P<0.039). Proteins contributed the most to the total energy content (Ria de Aveiro: Pearson, r=0.87, P<0.001; Ria Formosa Lagoon: Pearson, r=0.91, P<0.001)

(Table 2.2). Significant differences were observed between populations for total energy (K-W., H=3.90, df=1, P<0.05), especially in July and August 2010 and February 2011 and 2012. The total energy content only differ from 2010 to 2011 in May for Ria de Aveiro population (K-W., H= 4.34, df=1, P=0.037) and in July and August for Ria Formosa Lagoon clams (K-W., H=10.26, df=1, P=0.001).

2.4. Discussion

The reproductive activity of bivalves, which includes a sequence of events from gametogenesis to spawning, is controlled by the interaction between endogenous (Normand et al., 2008; Enríquez-Díaz et al., 2009) and exogenous factors, mainly food availability and temperature. Moreover, temperature is closely linked to the geographical locations affecting indirectly the availability of food and/or consequently the timing and duration of the reproductive cycle and number of spawning *per* year. For *R. decussatus* a different number of annual spawning has been described within Europe (da Costa et al., 2012).

Seawater temperature pattern, in this study, was common for both studied lagoons and was typical from temperate climates; increased during spring, stabilized in summer, decreased during autumn and remained low in winter. Nevertheless, Ria de Aveiro was characterized by relatively lower SST values than Ria Formosa Lagoon.

Despite no significant correlation was found between SST and the gonadal index, the sequence of gametogenic stages showed that the reproductive cycle of these two populations of *R. decussatus* follow a seasonal cycle, as has been previously reported for this and several other bivalve species (*e.g.* Gabbott, 1976; Xie and Burnell 1994; Albentosa et al., 2007). The reproductive cycle of *R. decussatus* comprised a ripe stage in spring followed by spawning that began in late spring and extended during summer until early autumn in both populations. A similar reproductive cycle was described in the studies by Ojea et al. (2004) and Breber (1980) for a population of *R. decussatus* from Galicia (Spain) and from Venice (Italy), respectively. However, other authors have shown the occurrence of two major periods of spawning, in spring and then in summer or early autumn in different populations of this species (Morocco - Shafee and Daoudi, 1991; France - Borsa and Millet, 1992; Lauruelle et al., 1994; Greece – Chryssanthakopoulou and Kaspiris, 2005) including in populations from the Ria Formosa Lagoon (Vilela, 1950; Pacheco et al., 1989). The differences observed between studies have been frequently associated to the influence of the geographical location and consequently by the inherent environmental factors (*vide* da Costa et al., 2012). Nevertheless, in our study, no significant differences were observed between the reproductive cycles of the two geographically distinct populations studied.

Table 2.3. Mean values (±SD, *n*=10) of proteins, glycogen, total lipids (µg mg^{-1} AFDW) and total energy (KJ g^{-1} AFDW) of *Ruditapes decussatus* during the experimental period.

		Ria de Aveiro				Ria Formosa Lagoon			
Year	Month	Protein (µg mg^{-1} AFDW)	Glycogen (µg mg^{-1} AFDW)	Total lipids (µg mg^{-1} AFDW)	Total energy (kJ mg^{-1} AFDW)	Protein (µg mg^{-1} AFDW)	Glycogen (µg mg^{-1} AFDW)	Total lipids (µg mg^{-1} AFDW)	Total energy (kJ mg^{-1} AFDW)
2010	May	531.7 ± 180.0	45.0 ± 10.6	46.1 ± 9.7	12.0	406.3 ± 113.6	42.0 ± 14.9	51.6 ± 18.1	9.9
	Jun	482.0 ± 98.0	37.7 ± 12.9	41.9 ± 25.0	10.8	388.5 ± 78.2	45.0 ± 9.3	27.2 ± 7.3	8.7
	Jul	465.4 ± 77.8*	32.0 ± 15.0	38.9 ± 9.6	10.3*	206.2 ± 105.3*	38.7 ± 9.3	37.4 ± 13.1	5.7*
	Aug	349.5 ± 101.0*	18.3 ± 4.2	45.6 ± 10.6	8.2*	157.4 ± 40.0*	28.6 ± 11.5	42.2 ± 8.9	4.8*
	Sep	128.3 ± 32.6*	53.7± 22.9*	53.5 ± 11.7	5.1	274.7 ± 67.5*	7.2 ± 2.4*	40.2 ± 9.1	6.5
	Oct	342.02± 37.2	34.6 ± 12.3*	35.0 ± 9.8	8.0	520.8 ± 123.5	13.3 ± 12.2*	41.3 ± 16.7	11.1
	Nov	--	--	--	--	--	--	--	--
	Dec	--	--	--	--	--	--	--	--
2011	Jan	--	--	--	--	--	--	--	--
	Feb	407.8 ± 69.4*	25.5 ± 7.0	47.3 ± 11.9	9.4*	142.2 ± 23.6*	22.1 ± 7.4	45.6 ± 11.0	4.5*
	Mar	301.2 ± 33.5	48.3 ± 15.6	97.6 ± 17.7	9.7	--	--	--	--
	Apr	--	--	--	--	--	--	--	--
	May	251.5 ± 33.4	33.2 ± 8.2	80.6 ± 18.1	7.9	284.5 ± 24.1	35.4 ± 8.9	93.0 ± 16.3	9.0
	Jun	270.5 ± 40.1	9.1 ± 5.8	113.5 ± 26.1	9.0	283.5 ± 52.0	14.5 ± 8.2	92.1 ± 18.8	8.6
	Jul	276.6 ± 38.2	23.1 ± 5.4	100.2 ± 35.5	8.9	293.2 ± 22.5	18.2 ± 4.1	112.1 ± 15.1	9.5
	Aug	265.4 ± 31.5	39.7 ± 12.8*	66.3 ± 12.2	7.8	349.2 ± 37.5	15.3 ± 2.3*	68.6 ± 12.2	9.0
	Sep	--	--	--	--	--	--	--	--
	Oct	298.7 ± 36.0	50.2 ± 13.4*	69.6 ± 13.4	8.7	393.7 ± 29.7	18.3 ± 4.1*	95.6 ± 14.1	10.8
	Nov	243.0 ± 76.7	50.7 ± 19.7*	62.3 ± 11.5	7.4	248.6 ± 62.7	21.7 ± 4.6*	82.5 ± 10.0	7.8
	Dec	--	--	--	--	--	--	--	--
2012	Jan	--	--	--	--	--	--	--	--
	Fev	297.1 ± 34.1	28.3 ± 23.1	59.4 ± 7.7	7.9*	475.4 ± 38.4	25.0 ± 13.0	90.9 ± 9.6	12.2*
	Mar	--	--	--	--	--	--	--	--
	Apr	235.1 ± 37.9	34.2 ± 12.6	118.1 ± 20.5	9.0	229.2 ± 37.7	35.2 ± 10.5	88.6 ± 15.2	7.9

(*statistically significant differences, $P<0.05$ found between populations)

Beside the fact that the seasonal pattern presented a spawning period well-defined in time, clams of the two studied populations exhibited an advantageous reproductive strategy for the species ensuring a consistent supply of gametes during the whole spawning period. Indeed, histological analyses showed simultaneously gonias, maturing gametocytes and variable proportions of fully matured gametes in the same individual, both in males and females. This high capacity for gonadal regeneration had also been previously observed by our team in *Venerupis senegalensis* from Ria de Aveiro (Joaquim et al., 2011). Nevertheless, and despite the intra-individual asynchrony, the maintenance of a synchronized gonadal development observed between males and females ensures the reproductive success of the species since sperm and oocytes will be expelled into the water column simultaneously during the spawning period, augmenting the probability of fertilization. This synchronism had previously been reported by Laruelle et al. (1994) and Ojea et al. (2004) for this species. Both populations showed a long reproductive rest phase that was extended by a period of approximately six months, during autumn and winter.

The onset of the gametogenic cycle with the proliferation of gonias was triggered by the rise of SST in late winter/early spring for both populations and the development of gametes intensified quickly until the attainment of the ripe stage late spring. These results were generally consistent with the previous findings by Vilela (1950), Shafee and Daoudi (1991) and Chryssanthakopoulou and Kaspiris (2005), in other populations of this species, although, as previously mentioned, these authors reported another onset of gametogenesis in July/August.

Condition index is generally considered to reflect the reproductive activity of bivalves (Fernández-Castro and Vido de Mattio, 1987; Massapina et al., 1999; Ojea et al., 2004). The positive correlation between these two parameters has been observed in several bivalve species from the Portuguese coast (*e.g.* Gaspar and Monteiro, 1988; Moura et al., 2008, Joaquim et al., 2011). In this study the CI did not followed the same trend in 2010 and 2011 and significant differences were also exhibited between populations, especially when clams were in the inactive stage (Period of sexual rest), between September and October 2010 and August and November 2011. Despite the fact that a positive correlation between CI and SST was observed in Ria de Aveiro, the CI did not reflect the reproductive cycle of this population, since no significant relationship was found between these parameters. In 2010, the CI of this population followed the SST increase and generally trended upwards until September. After that, the CI decreased, coinciding with the end of the spawning period and SST decrease. In 2011, Ria de Aveiro population showed two peaks in CI that accompanying the SST trend; the first one in May when the majority of the clams were ripe and another one in October, however, in this last one the rest stage was the more frequent stage observed.

In contrast, no significant positive correlation was found between CI and SST in the Ria Formosa Lagoon population, however, in this population, the CI was positively correlated with the GI. In 2010, CI remained high when the majority of clams were in advanced gametogenesis

and ripe stages, decreasing sharply with partial spawning and consequent rest period. In 2011, Ria Formosa Lagoon population only showed one peak of CI in May/June which corresponded to the maximum GI value of that year achieved with the contribution of the ripe stage of most of the clams. The reduction of the Ria Formosa Lagoon CI coincided with the rest period of clams.

In previous studies it has been reported that the CI is highly influenced by the energy storage and exploitation strategy of bivalve species (Joaquim et al., 2008; 2011; Delgado and Pérez-Camacho, 2005). Clams from Ria de Aveiro seem to have the ability to recover quickly the reserves after spawning when the SST and probably food availability are still high. These reserves seem to be harnessed to maintain their physiological state during winter. The same was not verified in the Ria Formosa Lagoon population. Clams lost their reserves with the intensification of the spawning event and were only able to recover them, slowly during winter, and more rapidly with the next SST increase. The major energetic effort suffered by clams during spawning leads then to their debility which might be at the origin of episodes of severe mortalities in the Ria Formosa Lagoon population after the reproductive period. Several factors can contribute to this difference in the energy storage and exploitation strategy between the two studied populations. da Costa et al. (2012) reports that another environmental variable that affects the energetic strategy of bivalves is immersion time. In our study, the subtidal clams from Ria de Aveiro showed higher CI values than the intertidal clams of Ria Formosa Lagoon during the rest period.

Several studies on bivalves have shown that sexual maturity is related to energy supply from previously stored reserves or the ingestion of available food and consequently is closely linked with the biochemical composition (Sastry, 1979; Pérez-Camacho et al., 2003). The reproductive cycle translates a seasonal pattern of biochemical composition that can vary among populations and species (Albentosa et al., 2007). The relative amounts of proteins (Ria de Aveiro - 128 to 532 µg mg^{-1} AFDW; Ria Formosa Lagoon – 142 to 520 µg mg^{-1} AFDW), glycogen (Ria de Aveiro - 9 to 54 µg mg^{-1} AFDW; Ria Formosa Lagoon - 7 to 45 µg mg^{-1} AFDW) and total lipids (Ria de Aveiro - 35 to 118 µg mg^{-1} AFDW; Ria Formosa Lagoon – 27 to 112 µg mg^{-1} AFDW) measured in *R. decussatus* were similar, in term of the proportions, to those previously described in the literature for this species (*e.g.* Ojea et al., 2004; Aníbal et al., 2011).

Several authors have suggested that, in bivalves, somatic proteins are used as an energy reserve in situations of nutritional stress and energy imbalance or during gonadal maturation (*e.g.* Gabbott and Bayne, 1973; Liu et al., 2008). Moreover, it has also been suggested that some species use proteins as a source of energy maintenance when carbohydrate reserves have already been depleted (Albentosa et al., 2007; Joaquim et al., 2011, da Costa et al., 2012). In this study, in 2010 the sudden decrease in protein content in both populations after spawning, when glycogen reserves were also depleted, suggests that proteins were indeed used for maintenance of the species; however, the same was not true for

2011 where no regular seasonal trend was observed. In this study, no correlation was found between proteins and glycogen and GI and significant differences were found between both populations protein content.

Glycogen is the main energy reserve in adult bivalves. In *R. decussatus* it can be an energy source for growth and at the same time stored in specific cells as an energetic reserve for the gametogenesis and gonadal development (Rodríguez et al., 1993). In this study, differences were observed between the two populations concerning glycogen content, although the rapid gonadal development and spawning process forced a striking consumption of the glycogen in both populations, after that, higher and lower values were recorded for Ria de Aveiro and Ria Formosa Lagoon, respectively. During the rest period the glycogen content of both populations also showed opposite trends. Some bivalve species store glycogen when food is abundant and gametogenesis takes place when there is low food availability, thus allowing the first spawning release when seawater temperature increases (conservative strategy), and there are others species in which the gamete production occurs from spring onwards, coupled with the first phytoplanktonic blooms (opportunistic strategy) (da Costa et al., 2012). Ojea et al. (2004) and Urrutia et al. (1999) in studies with Galician and Basque Country populations (Spain) have considered *R. decussatus* as a conservative species however Aníbal et al. (2011) concluded that *R. decussatus* from the Ria Formosa Lagoon exhibited an intermediate strategy. Our results corroborate this last thesis. In fact, although glycogen was positively correlated with GI, which is typical from an opportunistic species, the Ria Formosa Lagoon population stored this reserve during the winter and after that both stored and recently assimilated glycogen content was used for gametogenesis. In Ria de Aveiro population, despite the fact that clams have used the after spawning stored glycogen reserves during autumn and winter, which is typical from a conservative species, gametogenesis was not started and no significant correlations were found between glycogen content and GI. The onset of gamete production was only verified in early spring, probably associated with the first phytoplankton blooms, which is typical of an opportunistic strategy. These differences in the reproductive strategy between geographically distinct populations of the same species were also reported by da Costa et al. (2012) and Cerviño-Otero (2011). As previously mentioned the two populations also differed in the amount of glycogen stored, especially when clams were inactive in terms of reproduction. In fact, reserves were stored as glycogen soon after the spawning period in Ria de Aveiro population, however the same was not verified in the Ria Formosa Lagoon population. Consequently, in rest stage, the reserves of glycogen were considerably higher in Ria de Aveiro clams then in Ria Formosa Lagoon one's, corroborating the CI results. A significant positive correlation between glycogen content and CI was found in Ria Formosa Lagoon population.

Several authors (*e.g.* Beninger and Lucas, 1984; Ojea et al., 2004; Mouneyrac et al., 2008) have reported that lipid seasonal variations are inversely related with glycogen, due to the conversion of glycogen to lipids, biosynthesized during the formation of gametes (Gabbott, 1975). In the present study, total lipids increased with the onset of gametogenesis. After that

and during the reproductive period, an erratic variation of total lipids occurred in both populations that could be related with the successive gamete production and release, typical of a partial spawning species. Total lipids also peaked immediately after spawning. These results suggest that, more than a consequence of gametogenesis, the total lipids content also reflects the energy accumulation process and its consumption during bivalve somatic development, as has been previously reported by other authors (Albentosa et al., 2007; Joaquim et al., 2008; 2011). However, no correlation was found between total lipids and glycogen, moreover, total lipids content was substantially lower in 2010 than in 2011, in both populations, and no significant differences were found between populations.

The negative correlation found between proteins and total lipids in clams from Ria de Aveiro population reinforce the idea that proteins can be used as an energy reserve in stress situations. Moreover, in our study, protein was the constituent that most contributed to the total energy of *R. decussatus*.

In conclusion, the results of this study show that *R. decussatus* has a partial spawning period, during which occurs successive and simultaneous production of gametes and spawning. Moreover, it was also demonstrated that this species can adopt different energy storage depending on the geographic origin. Clams of both populations show a high reproductive effort that almost depletes its energy reserves; however, while the Ria de Aveiro population retrieves them immediately after spawning, the same is not verified in clams from the Ria Formosa Lagoon which leads to their consequent debilitation.

Both populations presented however viable broodstock for intensive hatchery production of juveniles and the extended spawning period of both *R. decussatus* populations has interesting implications for the implementation of profitable aquaculture. Moreover, the high gonadal regeneration capacity presented by this species coupled with its high gonadal development rate would provide larvae during most of the year without the need of performing extensive and expensive broodstock conditioning.

The global information obtained in this study on the gametogenic cycle and consequent energy storage will also allow the determination of the optimal reproductive time for artificial spawning induction for aquaculture production of this species.

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Chapter 3

Effect of geographic origin, temperature and timing of broodstock collection on conditioning, spawning success and larval viability of *Ruditapes decussatus* (Linnaeus, 1758)

Matias D., Joaquim S., Leitão A., Massapina C., 2009. Effect of geographic origin, temperature and timing of broodstock collection on conditioning, spawning success and larval viability of *Ruditapes decussatus* (Linnaeus, 1758). Aquaculture International 17:257-271.

Abstract

Culture of *Ruditapes decussatus* is clearly limited by the availability of seed, as this production proceeds almost exclusively from natural recruitment. Artificial spawning and larval rearing programs could provide an alternative source of spat. This study was designed to evaluate the effect of different conditioning temperatures on the broodstock maturation, spawning success and larval viability of two geographically (north and south of the Iberian Peninsula) distinct populations of European clam (*R. decussatus*) collected at different periods of the year in order to create "optimal" artificial spawning and larval rearing programs. Two batches of clams from each population were collected in October and February, and conditioned at 18±1 ºC, 20±1 ºC and 22±1 ºC. Of the three variables analysed the timing of broodstock collection was the most determining factor for gametogenic development, spawning and larval rearing. Geographic origin and conditioning temperature also greatly affected the spawning. The results also showed that the February conditioning was more effective than October and that the best conditioning temperatures were 20±1 ºC and 22±1 ºC for the northern and southern populations, respectively. These results suggest that the efficient conditioning temperature for each population of the same species is related to the seasonal temperature regime from their geographic origin. Larval viability and growth performance seemed to be independent of the broodstock conditioning.

Keywords: Artificial spawning; Conditioning; Broodstock; Spawning; Larval viability; Bivalves; *Ruditapes decussatus*.

3.1. Introduction

Aquaculture in Portugal is greatly supported by the European clam (*Ruditapes decussatus*) in Ria Formosa Lagoon (South Portugal), representing 34 % of the national aquaculture production and 80 % of the shellfish production (DGPA, 2006). This clam is commercially more important and more appreciated by consumers than the Japanese clam, *R. philippinarum*, production of which is forbidden in Portugal. Culture of *R. decussatus* is clearly limited by the availability of seed once this production proceeds exclusively from natural recruitment. In the last few years, the productivity of the Ria Formosa Lagoon has clearly decreased due to recruitment failures. To address this situation, artificial spawning and larval rearing programs could provide an alternative source of spat.

Artificial reproduction of bivalves requires the use of animals that have attained an optimal sexual condition, which according to Kennedy et al. (1996) depends on the synergetic effect of both internal and external factors. Most often temperature and food availability are considered the external key factors. Temperature has traditionally been assigned a major role, although the existence of sufficient nutritional reserves in the animal or the abundance of food also plays a major part in the evolution of the reproductive cycle (Pérez-Camacho et al., 2003). Therefore, a way to induce sexual maturation in bivalves is the manipulation of their physical and nutritional environments (Gallager and Mann, 1986). This process of artificially obtaining sexually mature individuals is called broodstock conditioning.

The conditioning required to bring broodstock to the optimum stage of gonadal development is dependent upon the stage of gonadal development at the beginning of conditioning and upon the conditioning rate (Lannan et al., 1980; Muranaka and Lannan, 1984; Andersen and Ringvold, 2000).

The response of bivalves to conditioning regimes varies widely among species. There is evidence that responses can also vary among different geographical populations in the same species as has been found for *Mytilus galloprovincialis* (Iglesias et al., 1996) and *Argopecten purpuratus* (Avendaño and Le Pennec, 1997). The differences in gonadal cycles and conditioning optima in different populations have to be considered in hatchery operations (Lannan et al., 1980; Devauchelle and Mingant, 1991). In the case of the European clam, in natural conditions, it has been reported that the ecotype decussatus living in different areas, even at the same latitude, could strongly differ in their fecundity levels and biochemical compositions (Shaffee and Daoudi, 1991; Trigui-El-Menif et al., 1995). Broodstock conditioning experiments carried out in our laboratory with three populations from different latitudes [Ria Formosa Lagoon (37º01′N; 07º49′W) in South Portugal, Ria Aveiro (40º42′N; 08º40′W) in North Portugal and the Rías Galegas (42°22′N, 8°56′W) in Northwest Spain] have systematically

showed difficulties concerning the induction of artificial spawning in the southern clam's population (unpublished data).

Under laboratory conditions many factors, including those affecting gametogenesis and broodstock conditioning, influence larval development in both early and late juveniles stages (Martínez et al., 2000). Le Pennec et al. (1998) pointed out that pectinid egg development and consequent larval production are extremely variable in hatcheries and that results are not reproducible from one year to the next. For *Crassostrea gigas*, Lannan et al. (1980) demonstrated that this variation is related to gonadal development of parental oysters and that this involved environmental and heritable components.

There is only a few published studies on the broodstock conditioning of the European clam (*e.g.* Delgado and Pérez-Camacho, 2002, 2005, 2007; Silva et al., 2002; Delgado et al., 2004; Hamida et al., 2004).

In this study we aimed to evaluate the effect of different conditioning temperatures (18±1 ºC, 20±1 ºC and 22±1 ºC) on the broodstock maturation, spawning success and larval viability of two geographically (north and south of the Iberian Peninsula) distinct populations of the European clam (*R. decussatus*) collected at different periods of the year (October and February) in order to create "optimal" artificial spawning and larval rearing programs.

3.2. Materials and Methods

Two experiments were carried out between October and January (October conditioning), and between February and May (February conditioning).

3.2.1. Collection of clams

The experiments were carried out using adult specimens of *R. decussatus* with a shell length >35 mm. Individuals were collected in October and February from two natural populations, one in the south [Ria Formosa Lagoon (37º01′N; 07º49′W), Portugal], and the other in the northwest [Rías galegas (42°22′N, 8°56′W), Spain] coasts of the Iberian Peninsula (Figure 3.1). We will refer to these populations as south and north, respectively.

3.2.2. Conditioning

Three groups of 120 animals from each location were placed in 25 l plastic tanks in a flow-through circuit containing natural seawater filtered through a 1 µm mesh. Water salinity was 33. North and south clams were conditioned at 18±1 ºC, 20±1 ºC and 22±1 ºC for both

October and February conditioning. Filtered seawater (1 µm) was heated using a heat exchanger with titanium plates and delivered to the tanks at a flow rate of 0.6-0.8 l min^{-1}.

A peristaltic pump was used continuously to add food to the circuit. The food ration consisted of $5x10^8$ cells $clam^{-1}$ day^{-1} of the microalgae *Isochrysis galbana* clone T-ISO and *Chaetoceros calcitrans* in a proportion of 1:1 in terms of size, which corresponds to 4 % of the dry meat weight in dry weight of algae (Utting and Millican, 1997).

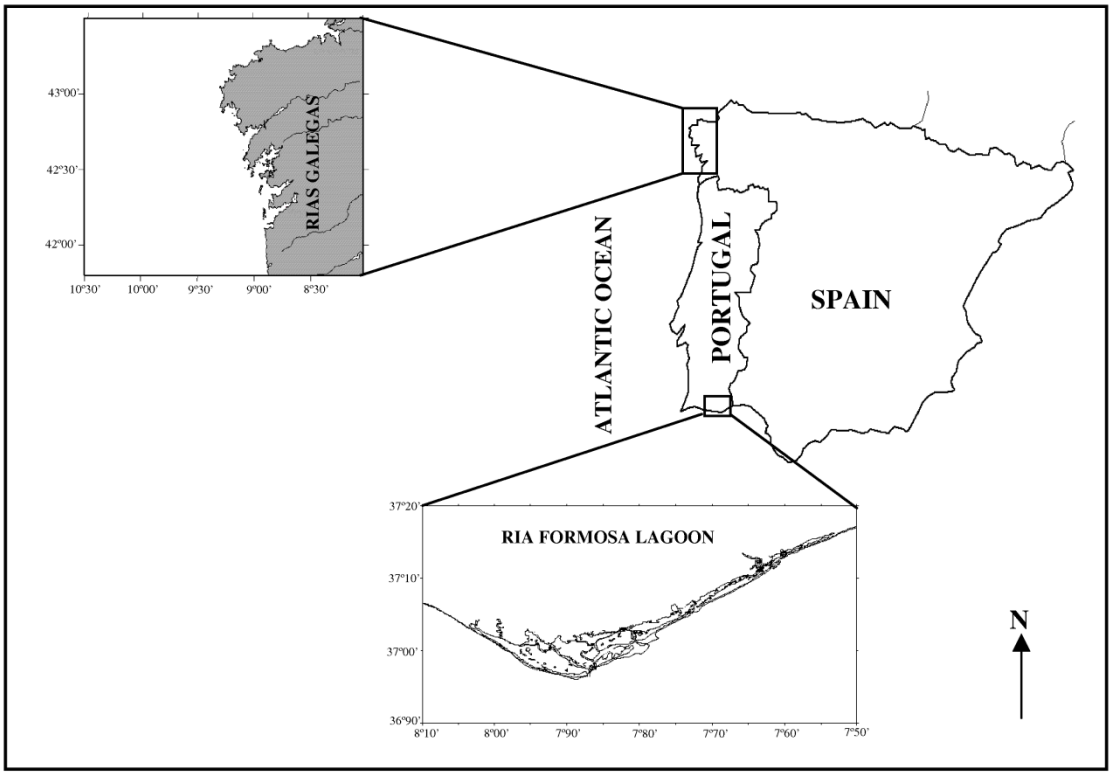

Figure 3.1. Collection locations of the two *Ruditapes decussatus* populations.

3.2.3. Broodstock sampling

The total conditioning period was of 11 weeks, with sampling performed at the beginning of the experiments (week 0) and at weeks 8 and 11 for the October conditioning and weeks 6, 9, and 11 for the February conditioning. At each interval (including the initial sample at week 0) two groups of eight individuals and one group of five individuals were sampled for determination of condition index, gametogenic condition and gross biochemical composition (glycogen and total lipids contents), respectively.

3.2.4. *Condition index and biochemical analysis*

The condition index of individual clams was calculated according to Walne and Mann (1975): [ash free dry weight (AFDW) of meat (g)/dry shell weight (g)]*100.

For each sample, the soft body was separate from the shell and both were put in an oven at 80 ºC and weighed after 24 h, then flesh was placed in a muffle furnace at 450 ºC for 24 h and reweighed to quantify ash.

Each determination of biochemical compounds (total lipids and glycogen contents) was carried out in duplicate on pooled material of five clams. Total lipids were extracted from fresh homogenised material in chloroform/methanol (Folch et al., 1957) and estimated spectrophotometrically after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966), and glycogen content was determined from dried (80 ºC for 24 h) homogenate using the anthrone reagent (Viles and Silverman, 1949). In both cases the results correspond to the mean of duplicate determinations and are expressed as a percentage of AFDW.

3.2.5. Determination of gametogenic condition

A conventional histology protocol was followed. The soft tissues were fixed in Bouin's fixative, subsequently trimmed, dehydrated in alcohol, cleared in xylene and embedded in paraffin wax. Sections 5-6 µm in thickness were stained with haematoxylin-eosin (Martoja and Martoja, 1967). Sections were classified according to Delgado and Pérez-Camacho (2005) (Table 3.1).

3.2.6. Spawning and larval rearing

After 11 weeks of conditioning, each broodstock treatment, which consisted of 50±5 clams, was induced to spawn by thermal stimulation, through a rapid increase of temperature from 20 ºC to 28±1 ºC over a 6 h interval. The oocytes were fertilised by addition of a mixture of sperm from various males. Embryos from each female and from each treatment were incubated in triplicate 5 l tanks, with 1 µm filtered and UV irradiated seawater, maintained at 20±2 ºC, at a density of 100 eggs per milliliter. After 48 h, the veliger rate (percentage of D-larvae) relative to the initial number of eggs was calculated based on three 1 ml aliquots. D-larvae were then reared until metamorphosis in triplicate 5 l tanks, using standard hatchery techniques (Walne, 1974). During larval rearing, water was renewed every 2 days. In this step, larvae were gently strained onto 50 µm Nitex netting, concentrated into 500 ml and homogenised. Three 1 ml sub-samples were removed for survival measurement and to estimate the percentage pediveliger

larvae (presence of foot and possible morphological alterations in the velum). These parameters were determined relatively to the initial number of eggs by female. After each water renewal change, all treatments received an equal volume of phytoplankton cells. The larval diet was constituted of microalgae *I. galbana* clone T-ISO and *C. calcitrans* provided at a concentration of 100 cells μl^{-1}, in a proportion of 1:1 based on size terms. Every 2 days, larvae size was recorded based on anterior-posterior measure (n=30). Growth rates were calculated as the slope (K) of the least-squares linear regression of the clam's larvae size versus time.

Table 3.1. Reproductive scale for *Ruditapes decussatus* proposed by Delgado and Pérez-Camacho (2005).

Stage	Histologic description
Period of sexual rest (phase I)	Gonadal follicles are absent and connective and muscular tissue occupies the entire zone from the digestive gland to foot. There is no evidence of gonadal development and sex determination is not possible.
Initiation of gametogenesis (phase II)	Follicles and gonadal acini begin to appear in females and males. They increase in size, and appear covered with oocytes in the growth phase in the females and with immature gametes (spermatogonia and spermatocytes) in the males.
Advanced gametogenesis (phase III)	The follicles occupy a large part of the visceral mass. The presence of muscular and connective tissue is reduced. At the end of this stage, characterised by intense cellular growth in females, the oocyte protrudes from the centre of the lumen, remaining attached to the wall via the peduncle. The abundance of free oocytes equals those attached to the wall of the follicle. In males, majority of the acini were full of spermatids and spermatozoids.
Reproduction period (phase IV)	Corresponding to the maturity of the majority of gametes. In the mature oocytes the rupture of the peduncle occurs, and the oocytes consequently occupy the follicular interior. In males, the gonadal acini mainly contain spermatozoids.

3.2.7. Statistical treatment of data

The effect of geographical broodstock origin (north and south), conditioning temperature (18±1 ºC, 20±1 ºC and 22±1 ºC) and timing of broodstock collection (October and February) was evaluated using the condition index, the number of spawners, the number of female spawners and larval viability as response variables.

For the condition index, a multifactorial analysis of variance (SAS Institute, 2000) was performed using only data obtained at the beginning of the experiment (week 0) and at the end

of it (week 11), since sampling periodicity during the October and February conditioning was not the same.

Maximum-likelihood analysis of variance (SAS Institute, 2000) was used in spawning success and two parameters were measured: the proportion of spawners in the total of stimulated clams and the proportion of spawning females in the total of spawners.

The data of larval viability (percentage D and pediveliger larvae and growth rate) was analysed by one-way ANOVA. If interaction is significant, Tukey honest significant difference (HSD) test, for post hoc comparison of means, was used. Arcsin transformation was used prior to statistical analysis of percentage D and pediveliger larvae data. This analysis was performed with the software STATISTICA for Windows (STAT SOFT INC., 1993). Results were considered significant at $P<0.05$.

3.3. Results

3.3.1. Condition index

The condition index of broodstock groups exposed to different temperatures is shown graphically in Figure 3.2. An increase of the condition index was observed for all treatments, when compared with the initial value. At the beginning of the experiments the condition index was lower in clams collected in February than in the October. The clams conditioned in February experiment attained significant lower condition index than clams conditioned in October experiment (ANOVA, $F=9.71$, $df=1$, $P=0.004$) (Table 3.2). The multifactorial ANOVA test showed no significant effect of origin and conditioning temperature on condition index (ANOVA, $P>0.05$), but their interaction term was marginally significant (ANOVA, $F=3.83$, $df=2$, $P=0.032$). This suggests that the effect of the conditioning temperature depends on the origin of the broodstock. As can be seen in Figure 3.2 it appears that northern clams performed better at 18±1 ºC and 20±1 ºC compared to 22±1 ºC, while southern clams have a better performance at 20±1 ºC and 22±1 ºC compared to 18±1 ºC.

Table 3.2. Condition index of the two clam populations (North and South) (mean±SD, $n=8$) conditioned at different temperatures, at the beginning and end of the October and February experiments.

Population	Weeks of conditioning	Experiment (timing of broodstock collection)					
		October			February		
		18±1 ºC	20±1 ºC	22±1 ºC	18±1 ºC	20±1 ºC	22±1 ºC
North	0	10.1±2.1	10.1±2.1	10.1±2.1	6.6±1.9	6.6±1.9	6.6±1.9
	11	14.5±1.4	14.8±2.3	12.2±1.1	12.2±2.0	10.6±1.1	11.4±2.2
South	0	8.3±0.9	8.3±0.9	8.3±0.9	6.1±1.7	6.1±1.7	6.1±1.7
	11	12.4±1.4	15.4±2.5	13.3±1.0	10.3±1.0	12.9±2.8	13.7±2.2

Average values ± standard deviations are shown ($n = 8$)

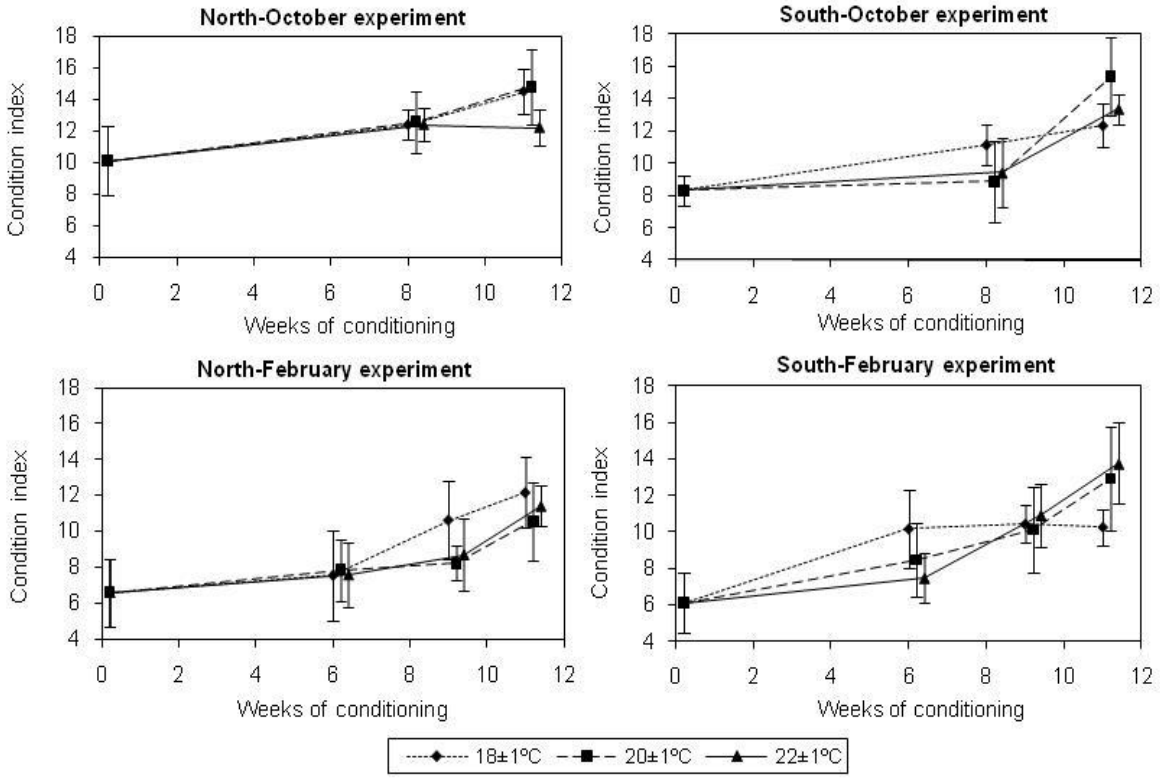

Figure 3.2. Condition index of the two clam populations (north and south) (mean±SD, *n*=8) conditioned at different temperatures, in October and February experiments.

3.3.2. Gonad development

Results of gametogenic stage in each experiment per origin are presented in Figure 3.3. The maturation process in the northern population with October conditioning was faster than the southern population for the same period and also faster than the February conditioning for both populations. At the beginning of the October experiment, the gonads of the southern and northern population contained different percentages of gonad development. Phase II (initiation of gametogenesis) was more marked in the northern population (80 %), while phase I (period of sexual rest) was more represented in the southern population (60 %). In the February experiment, the occurrence of phases I and II was very similar (≈ 50 %).

A clear effect of conditioning temperature on the clams' sexual maturation was observed through differences in the gonadal development rate. At 18±1 ºC, 8/9 weeks after the beginning of experimental period, most of the specimens were at phases II and III while those at 20±1 ºC and 22±1 ºC were at phases III and IV.

During both conditioning experiments, some heterogeneity among individuals within each population was observed, which is evident by the co-occurrence of initiation gametogenesis (phase II) and reproduction period (phase IV). This fact was particularly remarkable in the southern population.

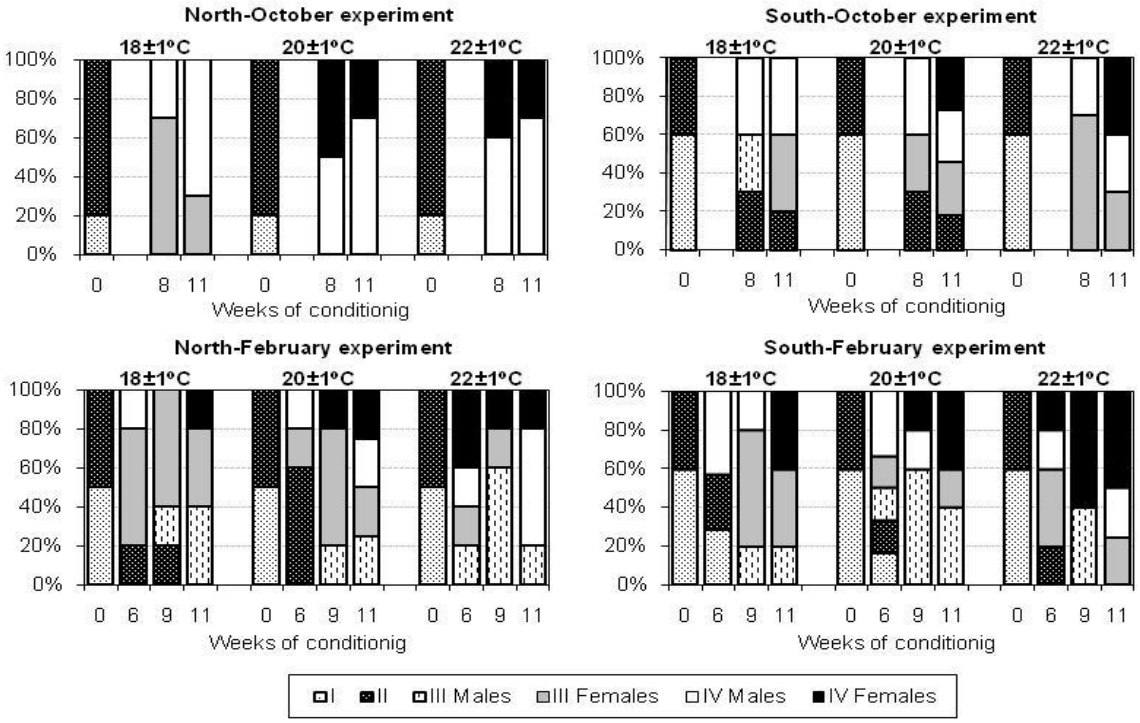

Figure 3.3. Gonadal development phases of the two clam populations (north and south) conditioned at different temperature, in October and February experiments.

3.3.4. Biochemical analysis

Values of the glycogen and total lipids contents of clams during conditioning are presented in Table 3.3, expressed as a percentage of dry weight. In general, differences in biochemical composition between populations were more apparent during the February conditioning than the October conditioning, although no clear pattern could be observed. In the October conditioning, fluctuations of glycogen and total lipids in southern clams at 18±1 ºC were similar to those observed in northern clams and the percentage of glycogen contents was higher than the total lipids until week 11. At 20±1 ºC and 22±1 ºC, inversion of the variation pattern of these parameters occurred after week 8 of conditioning. At the beginning of February conditioning, the northern population presented higher values of glycogen content than the southern population. At all temperatures, the general tendency of lipids content was to increase during the time of conditioning, although at 20±1 ºC and 22±1 ºC a slight decreased was observed after week 9.

Table 3.3. Total lipid and glycogen contents, as percentage of dry meat weight, in clam broodstock under different experimental conditions.

Population	Biochemical composition (% dry weight)	Weeks of conditioning	Experiment (timing of broodstock collection)					
			October			February		
			18±1 ºC	20±1 ºC	22±1 ºC	18±1 ºC	20±1 ºC	22±1 ºC
North	Glycogen	0	5.8	5.8	5.8	7.8	7.8	7.8
		6	-	-	-	7.2	9.2	10.7
		8/9	7.8	4.8	7.8	5.0	7.0	5.6
		11	7.4	6.3	2.3	3.1	5.5	2.1
	Total lipids	0	3.3	3.3	3.3	1.8	1.8	1.8
		6	-	-	-	6.0	6.7	6.2
		8/9	6.3	5.5	5.1	5.6	7.6	9.5
		11	8.1	7.9	7.6	12.0	6.2	5.4
South	Glycogen	0	6.6	6.6	6.6	4.7	4.7	4.7
		6	-	-	-	3.3	0.9	3.1
		8/9	7.7	6.2	4.4	7.4	5.6	3.6
		11	7.0	3.2	3.3	8.4	12.9	2.4
	Total lipids	0	3.3	3.3	3.3	3.2	3.2	3.2
		6	-	-	-	5.3	6.4	6.2
		8/9	6.2	7.9	6.1	9.3	7.5	8.6
		11	7.2	9.2	6.7	10.4	7.7	7.2

Values are mean of the two pooled sample of five clams.

3.3.5. Spawning and larval rearing

Spawning and larval viability results are presented in Table 3.4. The results of the log-likelihood analysis for the percentage of spawners show that the latter is influenced by the region of origin of the broodstock, the timing of broodstock collection and the conditioning temperature (M.-L., Chi^2=240.11, *df*=1, *P*<0.0001). The highest percentage of spawners was obtained with the northern clams during the February conditioning. In northern clams, the percentage of spawners varied from 9 to 71 % in the February conditioning and from 4 to 28 % in the October conditioning, depending on the conditioning temperature. In southern clams the ranges of percentage of spawners were 0 to 26 % in February conditioning and 0 to 6 % in October conditioning. Also, there is clearly a better performance of clams conditioned at 20±1 ºC and 22±1 ºC with respect to those conditioned at 18±1 ºC, for both times of broodstock collection and origins. Southern clams did not spawn at all when conditioned at 18±1 ºC.

The number of females among the spawners was always much lower than that of males, with an average of 6.4 % in the nine cases in which spawning was achieved (range 0-17 %) (Table 3.4). The statistical analysis indicates that the main factors that influenced the percentage of females spawners were origin (M.-L., Chi^2=7.22, *df*=1, *P*=0.007) and conditioning temperature (M.-L., Chi^2=8.74, *df*=2, *P*=0.013). The total percentage of female spawners was higher in the February conditioning (37 %) than in the October one (21 %).

Data on the average number of eggs released by females are too scarce to allow the observation of a general trend (Table 3.4). There is a very high variability in the recorded values which range from 0.02 million eggs female^{-1} obtained in northern clams conditioned at 22±1 ºC in the October experiment, to 3 million eggs female^{-1} released by clams from the south conditioned at 22±1 ºC, also in the October conditioning.

The larvae obtained from northern clams conditioned at 22±1 ºC in the October experiment and from southern clams conditioned at 22±1 ºC in the February conditioning were not viable. For the other four combinations of origin, timing of broodstock collection and conditioning temperature that produced eggs the percentage of D-larvae and growth rate were very similar (ANOVA, *P*>0.05). The highest percentage of pediveliger larvae was observed in the February conditioning for the southern population conditioned at 20±1 ºC (55 %) and the lowest rate was found in the October conditioning for the southern population conditioned at 22±1 ºC (31 %) (Table 3.4). The clams (southern and northern origins) conditioned at 20±1 ºC in the February conditioning presented a higher percentage of pediveliger rate compared to the southern clams conditioned at 22±1 ºC in the October experiment (ANOVA, *F*=9.83, *df*=1, *P*=0.002).

Table 3.4. Spawning characteristics and larval viability under the different experimental treatments.

Population	Spawning and larval parameters	Experiment (timing of broodstock collection)					
		October			February		
		18±1 ºC	20±1 ºC	22±1 ºC	18±1 ºC	20±1 ºC	22±1 ºC
North	No. of clams	49	47	47	53	52	45
	Spawners (%)	4	15	28	9	71	69
	Female spawners (%)	0	0	17	0	17	16
	Mean no. eggs released (10^6)	-	-	0.02	-	1	0.23
	D-larvae (%)	-	-	0	-	96	84
	Pediveliger larvae (%)	-	-	-	-	47	42
	Growth rate	-	-	-	-	5.08	4.49
South	No. of clams	48	47	51	55	53	47
	Spawners (%)	0	6	4	0	8[a]	26[a]
	Female spawners (%)	0	0	4	0	[a]	4[a]
	Mean no. eggs released (10^6)	-	-	3	-	0.34	0.48
	D-larvae (%)	-	-	85	-	93	0
	Pediveliger larvae (%)	-	-	31	-	55	-
	Growth rate	-	-	4.72	-	4.65	-

[a] Spontaneous spawning

3.4. Discussion

The results obtained in this study demonstrate the importance of the timing of broodstock collection, geographic origin and temperature during the hatchery broodstock conditioning of *R. decussatus*. Of the three variables analysed, the timing of broodstock collection was the most determining factor on gametogenic development (gonadal stages and condition index), spawning and larval rearing. Geographic origin and conditioning temperature greatly affected the spawning.

The gonadal development and the condition index of clams were considered as the key parameters of the sexual maturation process (*e.g.* Walne and Mann, 1975; Laruelle et al., 1994; Rodriguez-Moscoso and Arnaiz, 1998; Ojea et al., 2004). The gonadal phases I and II (sexual rest and initiation of gametogenesis) of broodstock animals at the onset of both experiments (October and February) was consistent with the observations in the wild for the same periods concerning the reproductive cycles for the northern wild population (Rodriguez-Moscoso and Arnaiz, 1998; Ojea et al., 2004) and with those of Vilela (1950) and Pacheco et al. (1989) for the southern wild population. The maturation process was faster in the northern population in the October experiment than in the other conditions tested. This may be due to the fact that this population was already in a more advanced gametogenesis process at onset of the October experiment, supporting that gonadal stage at the beginning of conditioning is crucial to the development of broodstock conditioning process. The occurrence of advanced gametogenesis and reproduction period phases was dependent on conditioning temperature, since higher temperature accelerated gametogenesis development; our observations are congruent with Delgado and Pérez-Camacho (2007), also in *R. decussatus*.

The effect of timing of broodstock collection was evidenced by the comparison of the condition index values obtained at the end of each conditioning. Clams which initiated conditioning in October reached higher condition index than those that initiated in February. This may be due to the fact that this parameter already had higher values at the beginning of the October experience. A strict relationship between the condition index increments and the gonadal development was frequently observed by several authors (*e.g.* Lucas and Beninger, 1985; Hamida et al., 2004; Ojea et al., 2004; Mladineo et al., 2007). Thus, the existence of significant differences between the time that conditioning began revealed that the state of the clams at the beginning of the conditioning was determining. The effect of the conditioning temperature on the condition index depended on the origin of the broodstock; the northern clams performed better at 18±1 ºC and 20±1 ºC compared with at 22±1 ºC, while southern clams exhibited better performance at 20±1 ºC and 22±1 ºC compared with at 18±1 ºC. Therefore, the different performance in terms of condition index found in the northern and southern populations in the laboratory seemed to be related to the seasonal temperature

regime in the two localities of origin, although a possible different genetic adaptation of the populations should also be considered. In the north, the annual temperature of seawater varies between 7 ºC and 19 ºC (Pérez-Camacho, 1980) while in the south it varies from 14 to 25 ºC, with the highest temperatures (21±4 ºC) from May to November (Falcão, 1997).

It is generally accepted that gametogenic cycle is closely linked to the seasonal cycle of storage and utilisation of glycogen and subsequent *de novo* synthesis of lipids during vitellogenesis at the expense of stored glycogen (Gabbott, 1975; Gallager and Mann, 1986). Several authors have reported maximum glycogen content in bivalves immediately preceding and during gamete proliferation (Ansell et al., 1980; Barber and Blake, 1985; Ojea et al., 2004). The range of values of glycogen and total lipids content of the present study are in agreement with the range observed by Ojea et al. (2004) for *R. decussatus* during gametogenic cycle, although no clear general pattern was observed.

The northern clams spawned much more often than southern clams in both the October and February experiments. Our finding of a greater response to spawning induction in northern compared to southern population was probably associated with the environmental conditions of origin, especially temperature, and with the spawning induction method. The method used for spawning induce was thermal stimulation, with a rapid increase of temperature from 20 to 28±1 ºC. Since the southern population is perfectly adapted to high temperatures and to these variations in nature, the protocol used for thermal stimulation was probably not the most adequate for the southern clams, since it is necessary to try other ranges of temperature, length of stimulation and rapidity in temperature increase, or other kinds of stimulation.

There was an evident effect of the conditioning temperature on the response of clams to spawning induction. Better performance was clearly exhibited by clams conditioned at 20±1 ºC and 22±1 ºC compared with those conditioned at 18±1 ºC, in both experiment and for both populations. Many authors (Sastry, 1975; Laruelle et al., 1994; Pérez-Camacho et al., 2003; Delgado and Pérez-Camacho, 2007) have reported that gametogenesis and spawning in bivalve molluscs are closely related to seasonal changes in seawater temperature. The February conditioning was more effective than the October conditioning in terms of spawning success. A possible explanation for this is that the clams in the February conditioning may have had more time in resting period than clams conditioning in the October experiment and were less affected by the last natural reproduction. In the present study no general trend was observed in the mean number of eggs released and in the viability and growth of larvae obtained from different treatment in response to spawning. These data seem to indicate that larval viability and larval growth performance are independent of the broodstock performance. However, we found significant differences in pediveliger rate, suggesting that these differences were probably due to the larval rearing conditions.

The present study also showed that the gametogenic stage and condition index are good indicators of the mean condition in order to evaluate the potential of a

population to be effectively conditioned, although due the heterogeneity characteristic of bivalves this must be done in a representative sample. On the other hand, the number of eggs released is not a good measure for successful conditioning of broodstock because a low percentage of veligers and relatively few larvae can result even if the number of eggs released is relatively high. The northern population seems to be more appropriate source of genitors for hatchery aquaculture, using thermal induction for spawning. It is possible, however, that the southern population could respond differently using a different protocol of thermal induction or a different method of spawning induction.

We believe that the results of our study could then be useful to *R. decussatus* hatchery production programs. These programs should take into consideration that: the general condition of animals before exposure to experimental conditions determines the success of broodstock conditioning and consequently the effective response of spawning induction; the best conditioning temperatures for northern and southern Iberian Peninsula populations were 20±1 ºC and 22±1 ºC, respectively; the efficient conditioning temperature for each population of the same species is related to the seasonal temperature regime from the geographic origin.

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Chapter 4

Hatchery broodstock performance of the European clam *Ruditapes decussatus* (Linnaeus, 1758): Influence of different diets and temperatures

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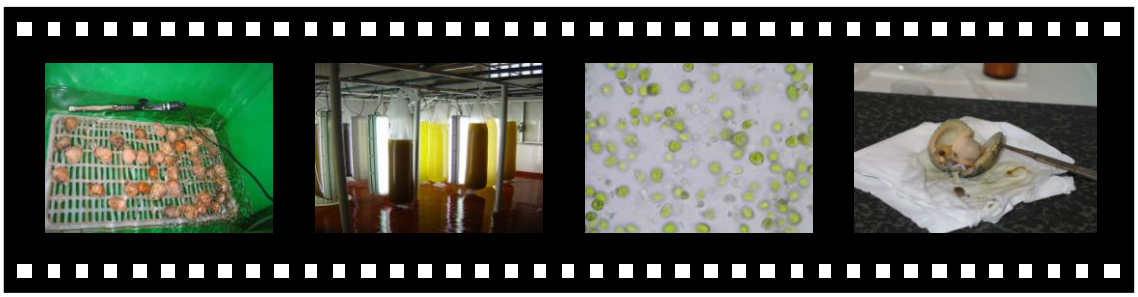

Abstract

The European clam, *Ruditapes decussatus*, is a species of high social and commercial interest to Southern European aquaculture. However the development of *R. decussatus* culture has been limited by the scarcity of natural recruitment and the current high cost of producing spat in commercial hatcheries. This species can indeed be considered an emerging species for which there is a demand in technological development for seed hatchery production. For hatcheries to consistently produce spat it is essential to develop broodstock conditioning techniques. Manipulate the gonadal cycle and spawning period of the clams so that adults can be spawned earlier or later than occurs in the natural environment is crucial. Food and temperature are the main factors that regulate the timing and rate of energy storage and reproduction in bivalves. This study was designed to evaluate the effects of different diets and temperatures on reproductive output of *R. decussatus* and express the evolution of the different lipid classes during sexual maturation, aiming to find a broodstock conditioning diet and temperature that maximizes fecundity and egg quality and that could be suitable for commercial hatcheries. Four groups of broodstock clams were conditioned at 20±1 ºC, under four nutritional regimes: unfed, two monospecific microalgae, *Isochrysis galbana* clone T-ISO and *Chaetoceros calcitrans* and, a mixture of *I. galbana* clone T-ISO and *C. calcitrans*. Another group of clams was conditioned at 22±1 ºC and was fed a mixture of *I. galbana* clone T-ISO and *C. calcitrans*. The gametogenesis process, energy storage and spawning success were all influenced by the nutritional value of the diet received, as evidenced by the differences in reproductive effort among the single and combined supplemental diets. This study demonstrates that the combination diet at 20 ºC is the most profitable to the conditioning of *R. decussatus* broodstock. Temperature was proven to accelerate the reproductive conditioning and it was put in evidence that the time necessary to complete gametogenesis is inversely correlated with temperature; however this was not reflected in the reproductive output. Two main conclusions emerge from the present study. i) temperature is a parameter that must be carefully managed to improve the reproductive conditioning of bivalves: high temperature throughout gametogenesis shorten the time to full ripeness but do not produce better reproductive output; ii) lipids are clearly of major importance in conditioning, either as energy reserves or as precursors of tissue structures being triacylglycerols the ones who play the major role.

Keywords: Bivalve; *Ruditapes decussatus*; broodstock conditioning; biochemical composition; diet; temperature.

4.1. Introduction

The European clam, *Ruditapes decussatus*, is a species of significant social and commercial interest for European aquaculture. However, the development of *R. decussatus* culture has been limited by the scarcity of natural recruitment and the high cost of producing spat in commercial hatcheries (Fernández-Reiriz et al., 1999; Matias et al., 2009). The European clam can indeed be considered an emerging species for which there is a demand in technological development for seed hatchery production, in contrast with the introduced Manila clam *R. philippinarum*.

Despite academic knowledge shown through scientific publications until now, progress in bivalve aquaculture has relied mainly on empirical approaches and this is particularly true for bivalve hatcheries, since this activity has a relatively recent (30 years) development. Moreover, the factors limiting the development of hatcheries/nurseries have never been considered systematically. The aquaculture industry depends on the availability of high quality juveniles, which will grow rapidly to commercial size. For hatcheries to consistently produce spat it is essential to develop broodstock conditioning techniques (Lannan et al., 1980; Pronker et al., 2008). Manipulate the gonadal cycle and spawning period of the clams so that adults can be spawned earlier or later than occurs in the natural environment is crucial (Ojea et al., 2008).

Reproduction of intertidal bivalves is an energy consuming process which includes gametogenesis, development and metamorphosis (Martínez et al., 2000). The success of this process depends on the physiological condition, especially the pre-spawning condition of the adult (Hendriks et al., 2003).

The combined effects of food and temperature, the main factors that regulate the timing rate of energy storage and reproduction in bivalves are complex and depend specifically on acquisition and expenditure of energy (Pérez-Camacho et al., 2003).

Previous studies have showed that increasing the water temperature initiates gametogenesis in bivalves, but that fecundity is mainly influenced by the quality and quantity of the algal diet (Lubet, 1976; Utting and Millican, 1997). The diet provided to adults can affect the biochemical composition of their gonads and their resulting eggs and larvae. Until now no commercial formulated diet for bivalves is available and hatcheries mostly rely on the use of microalgae (*e.g.* Muller-Fuega, 2000; Brown, 2002; Pronker et al., 2008). Feeding bivalves with a mixture of microalgae has became common practice in hatcheries since microalgae's species vary considerable in their nutritional value and seems that optimal food conditions can only obtained by mixing species (Utting and Millican, 1997; Brown, 2002). However in *R. decussatus* the few studies on nutrition developed on the broodstock conditioning of this species mostly considered monospecific diets (*e.g.* Delgado et al., 2004; Delgado and Pérez-Camacho, 2005).

In general, changes in each biochemical component are closely linked to the state of sexual maturity of the bivalve, and are related to energy supply either directly from the ingested food or from previously stored reserves (Navarro et al., 2000; Pérez-Camacho et al., 2003). The gross biochemical composition of the diet influences the physiology of bivalves, particularly if a major component is lacking. However, the composition of the specific forms of those major components (proteins, carbohydrates and lipids) can also be of influence (Labarta et al., 1999; Matias et al., 2009; Joaquim et al., 2011).

Lipids are usually used as an energy source during gametogenesis (*e.g.* Holland, 1978; Delgado et al., 2004), and constitute the principal nutritional reserve in eggs and larvae, conditioning their viability (Helm et al., 1973; Matias et al., 2011). Despite the few studies in this species (*e.g.* Delgado et al., 2004), it is clear that certain bivalves accumulate neutral lipids during gametogenesis (Soudant et al., 1996). Phospholipids and triacylglycerols appear to constitute the greatest fraction of vitellinic lipids in marine invertebrates (Holland, 1978; Delgado et al., 2004), whereas esters sterol show behavioural trends (Pazos et al., 1996; Soundat et al., 1996; Delgado et al., 2004).

In this study we describe the effects of different diets and temperatures on the reproductive output of *R. decussatus* and express the evolution of the different lipid classes during sexual maturation, aiming to find a broodstock conditioning diet that maximizes fecundity and egg quality and suitable to be used in commercial hatcheries.

4.2. Materials and Methods

4.2.1. Experimental setup

Adult clams, *R. decussatus* (35 mm ≤ total length ≤ 45 mm), were collected from a natural stock in Ria Formosa Lagoon (37º01′N; 07º49′W) (Portugal). They were divided randomly into five conditioning groups. The experiments were performed using 110 adult specimens in 25 L tanks containing natural seawater filtered through 1 µm, in a flow–through circuit at a rate of 0.8 l min^{-1}, in triplicate. Conditioning temperatures were chosen according to a previous study by Matias et al. (2009). Four groups of broodstock clams were conditioned at 20±1 ºC, under four nutritional regimes: unfed (starved), two monospecific microalgae, *Isochrysis galbana* clone T-ISO [T-iso (20 ºC)] and *Chaetoceros calcitrans* [C.cal (20 ºC)] and, a mixture of *I. galbana* clone T-ISO and *C. calcitrans* [T-iso+C.cal (20 ºC)]. Based on the general recognition that a mixture of microalgae constitutes a better nutritional regime for bivalves (Brown, 2002; Utting and Millican, 1997), another group of clams was conditioned at 22±1 ºC and supplied with a mixture of *I. galbana* clone T-ISO and *C. calcitrans* [T-iso+C.cal (22 ºC)].

The food was added to the circulation water by means of a variable-flow peristaltic pump, in a ration of 4 % of the clam dry meat weight in dry weight of microalgae (Utting and Millican, 1997). The mixed diet was constituted in a proportion of 1:1 dry weight of T-iso and C.cal. In order to maintain constant the food ration in each experiment, total food varied as the experiment progressed and clams were removed in successive samplings. The total conditioning period was of eight weeks, and sampling was performed at the beginning and at the weeks four and eight of the experiment. At each sampling time, three groups of 10 individuals were randomly selected for histological study of gonadal development and to determine condition index, total lipids, lipids classes and glycogen.

The microalgae T-iso and C.cal were batch cultured in 80 l plastic bags. The filtered (1-µm), UV-treated seawater (salinity 35) was chlorinated (0.1 ml l^{-1}) for 24 h, neutralized with 0.1 g l^{-1} thiosulfate and enriched with Walne medium before inoculation. Continuous aeration was provided to enhance growth and prevent the algae from settling. Microalgae were grown under 24 h light conditions, at a temperature of 20±1 ºC, and harvested daily in the exponential growth phase. Before being used as food, algal densities were determined daily by standard algal cell counts (Büker chamber). Biochemical composition analysis of the microalgae T-iso and C.cal was performed at the beginning and at the middle of the conditioning period. Samples were centrifuged, resuspended with 0.5 M ammonium formate, stored at -20 ºC and freeze-dried prior to biochemical analysis (proteins, carbohydrates, total lipids, polar lipids, neutral lipids and organic matter).

4.2.2. Spawning and larval rearing

The clams remaining in each tank at the end of the experiment, (53±8 individuals), were induced to spawn by thermal stimulation, through a rapid increase of temperature from 20 ºC and 22 ºC (according to the conditioning temperature) to 28±1 ºC at each interval of 2 h, over a 6 h interval. Three samples of 50.000 oocytes were taken for biochemical analysis from a pool of all oocytes by treatment. Also, 50 oocytes from each female (n=3) by diet were taken to evaluate the diameter. The oocyte diameter was grouped into four diameter classes: [47-54]; [55-62]; [63-70] and [71-78] µm.

From each treatment, gametes from all females were pooled and fertilized by addition of a mixture of sperm from males of the same treatment at a ratio of about ten spermatozoa to each oocyte. Embryos from each treatment were incubated in triplicate 5 l tanks, with 1-µm filtered and UV-irradiated seawater, maintained at 20±2 ºC, at a density of 100 eggs per ml. After 48 h of incubation, the D-larvae were collected on a 30-µm mesh screen and the veliger rate (% of D-larvae) relative to the initial number of eggs was calculated based on three 1 ml aliquots.

4.2.3. Histology

A conventional histology protocol was followed. Tissues were fixed in Bouin's fluid; 5-6 µm sections were prepared and stained with haematoxylin-eosin (Martoja and Martoja, 1967). Sections were classified according to Delgado and Pérez-Camacho (2005): Phase I- Period of sexual rest; Phase II- Initiation of gametogenesis; Phase III- Advanced gametogenesis; Phase IV- Reproduction period.

4.2.4. Condition index

The condition index of individual clams was calculated according to Walne and Mann (1975): [ash free dry weight (AFDW) of meat (g)/dry shell weight (g)]*100.

For each sample, the adductor muscles were cut and the clams placed on their ventral surface, to drain for 5 min. The soft tissues were separated from the shell and both were put in an oven at 80 ºC and weighted after 24 h, then the flesh was ashed in a muffle furnace at 450 ºC for 24 h and re-weighted.

4.2.5. Biochemical composition of adults, oocyte and microalgae

In the adults, each determination of the different biochemical compounds (glycogen, total lipids and lipids classes) was carried out in triplicate on pooled material from ten clams'. Glycogen content was determined from dried (80 ^{o}C for 24 h) homogenate using the anthrone reagent (Viles and Silverman, 1949). Total lipids were extracted from fresh homogenised material in chloroform/methanol (Folch et al., 1957). Lipids extracts of tissues were fractionated in two aliquots to analyse total lipids and lipid classes. Total lipids were estimated spectrophotometrically after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966). In both cases the results correspond to the mean of triplicate determinations and are expressed as a percentage of dry weight (% of DW).

One aliquot of the total lipids extracted was separated on the S-III Chromarods using four solvent systems according to the method developed by Parrish (1987). Lipid classes were quantified by the flame ionisation detection (FID) system of the Iatroscan Mark-V analyser (Iatron Laboratories Inc., Tokyo, Japan). The lipid classes detected were triacylglycerol, phospholipids, sterols, free fatty acid and sterol ester + waste. The results are expressed as a percentage of total lipids.

Each sample of oocyte and microalgae for biochemical analysis was rinsed with iso-osmotic ammonium formate (0.5 M) to remove salt, stored in liquid nitrogen, and then freeze-dried.

A micro-analytical fractionated extraction scheme developed by Holland and Gabbott (1971) and Holland and Hannant (1973) was followed for the determination of the contents of the different biochemical components. Lyophilized samples were homogenized in 500 µl distilled water using a sonicator. A separate sample (200 µl) of the initial homogenate was taken for analysis of total lipids, neutral lipids and another separate sample (200 µl) was taken to determine proteins and total carbohydrates.

Total lipid content was extracted by the method of Bligh and Dyer (1959) and taken up in 500 µl of chloroform. Total lipids were determined by the methods of Marsh and Weinstein (1966) using tripalmitin as a standard and the absorbance determined at 375 nm. Neutral lipids were determined in the same way as total lipids, 200 µl samples of neutral lipids, in chloroform, were dried for 20 min at 100 ºC and used for determinations. Polar lipids were determined as the difference between total and neutral lipids.

Proteins were precipitated by cold 5 % trichloroacetic acid (TCA) and the precipitate washed in warm 1.0 N NaOH. Protein concentration was assayed as described by Lowry at al. (1951) method, modified by Bensadoun and Weinstein (1976) and Hess et al. (1978) at 750 nm using serum albumin as a standard.

Hydrolysed and unhydrolysed samples of TCA supernatant were used for the determination of total carbohydrates and free reducing sugars by a modification of the method of Folin and Malmros (1929). The components were quantified with a ferricyanate reduction reaction at 420 nm using glucose as a standard. Polysaccharides were determined as the difference between carbohydrates and free reducing sugars.

The organic matter was the sum of proteins, total lipids and carbohydrates.

4.2.6. *Statistical analysis*

Condition index, biochemical composition of microalgae, adults and oocytes, number of oocytes release and veliger rate were examined by analysis of variance (ANOVA) or Kruskal–Wallis ANOVA on ranks, whenever the assumptions of ANOVA failed, among days of the same treatment and among treatments. Multiple pair comparisons among means were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test. Percentage data were arcsine transformed to normalize variance (Sokal and Rohlf, 1981). Results were considered significant at $P<0.05$. The statistical analyses were undertaken using the SIGMASTAT 3.11 statistical package.

4.3. Results

4.3.1. Biochemical composition of the microalgae

The biochemical composition and organic matter of the microalgae is presented in the Table 4.1. The microalgae T-iso presented significantly higher proportions of proteins (ANOVA, F=42.60, df=1, P<0.001), total lipids (K–W, H=8.31, df=1, P=0.002), neutral lipids (K–W, H=7.41, df=1, P=0.004) and organic matter (ANOVA, F=176.14, df=1, P≤0.001), while the microalgae C.cal presented a significantly higher proportion of carbohydrates (ANOVA, F=74.28, df=1, P<0.001).

Table 4.1. Biochemical composition (mean±SD, n=6) and organic matter of the microalgae *Isochrysis galbana* clone T-ISO and *Chaetoceros calcitrans*. Proteins, carbohydrates, total lipids, polar lipids, neutral lipids and organic matter are expressed in pg by cell.

	Microalgae biochemical composition					
	Proteins pg.cell^{-1}	Carbohydrates pg.cell^{-1}	Total lipids pg.cell^{-1}	Polar lipids pg.cell^{-1}	Neutral lipids pg.cell^{-1}	Organic matter pg.cell^{-1}
Isocrhysis galbana (T-ISO)	5.05±0.74	1.30±0.19	3.32±0.23	1.44±0.29	1.83±0.58	10.29±0.56
Chaetoceros calcitrans	2.65±0.42	2.57±0.31	1.83±0.39	1.07±0.55	0.83±0.20	7.63±0.93

4.3.2. Gonadal development

At the beginning of the experiment, the clams were either in sexual rest stage (stage I) or initial phase of gametogenesis (stage II), whereas mature individuals were absent (Figure 4.1). Afterwards, while the exception of the starved treatment, the gonadal development advanced rapidly under the experimental conditions and after 4 weeks, 60 % of the individuals from the monospecific diets T-iso and C.cal at 20 ºC showed signs of full reproduction (stage IV). For treatments T-iso+C.cal (20 ºC) and T-iso+C.cal (22 ºC), the corresponding values were 55 % and 90 %, respectively. After 8 weeks, the proportion of sexually mature individuals varied between 70 % for C.cal (20 ºC) and 100 % for T-iso+C.cal (20 ºC). For treatments T-iso (20 ºC) and T-iso+C.cal (22 ºC) the corresponding values were 90 % and 95 %, respectively. The clams maintained in starvation never attained full reproduction.

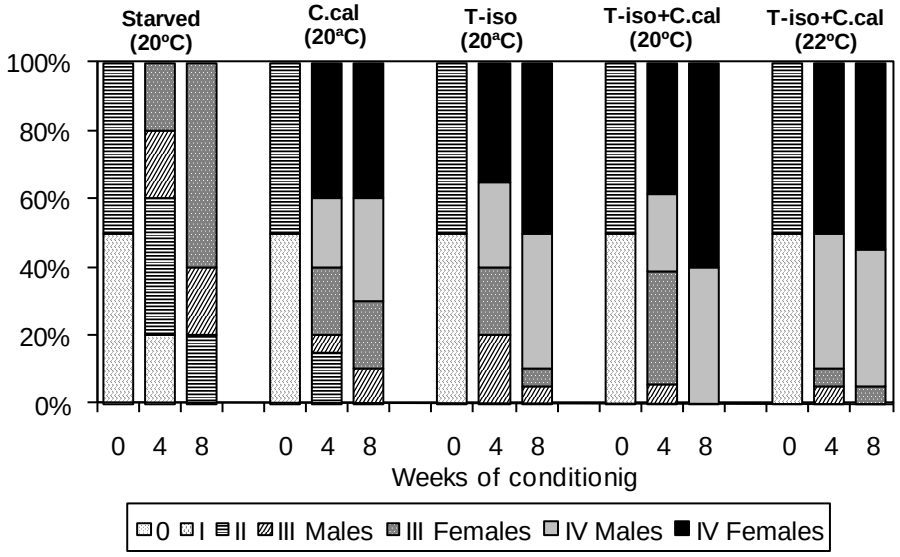

Figure 4.1. Gonadal development phases of the five broodsotck *Ruditapes decussatus* conditioned at different food regimes and temperatures: unfed (Starved); *Isochrysis galbana* clone T-ISO (T-iso); *Chaetoceros calcitrans* (C.cal); *I. galbana* clone T-ISO and *C. calcitrans* (T-iso+C.cal) all at 20 ºC and *I. galbana* clone T-ISO and *C. calcitrans* [T-iso+C.cal (22ºC)] at 22ºC.

4.3.3. Condition index

The evolution of the condition index of broodstock groups exposed to different diets and temperatures is presented in Figure 4.2. The condition index of fed clams increased throughout over the experimental period, whereas in clams held in starvation the condition index decreased from the first sampling time (T_0 – 8.1±1.5) to the second (T_4 – 4.9±1.2) and then remained stable (T_8 – 5.0±0.7). At the end of the conditioning period, values of condition index were higher for clams conditioned with the diet T-iso+C.cal (20 ºC) (19.5±2.0), followed by the treatments T-iso+C.cal (22 ºC) (16.6±1.5), T-iso (20 ºC) (13.9±2.2) and C.cal (20 ºC) (12.2±1.9). At this time, significant differences were observed between treatments [Starved ≠ (T-iso (20 ºC) = C.cal (20 ºC)) ≠ T-iso+C.cal (20ºC) ≠ T-iso+C.cal (22ºC)]. The two-way ANOVA test showed significant effect of diet/temperature (ANOVA, F=99.09, df=4, P≤0.001) and sampling times (ANOVA, F=184.49, df=2, P≤0.001) on the condition index and their interaction term was marginally significant (ANOVA, F=38.64, df=8, P≤0.001). In general, condition index was related to the stage of clam sexual maturation (see Figure 4.1), and was clearly greater in the individuals in the reproductive phase (T4 and T8), than in those sexually indistinguishable or at the beginning of the gametogenesis.

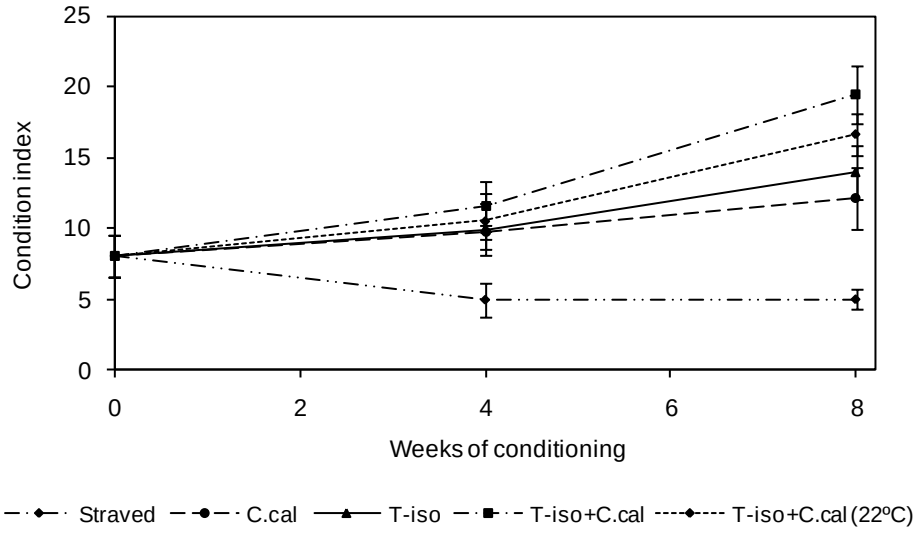

Figure 4.2. Condition index (mean±SD, *n* =10) of the five broodsotck *Ruditapes decussatus* conditioned at different food regimes and temperatures: unfed (Starved); *Isochrysis galbana* clone T-ISO (T-iso); *Chaetoceros calcitrans* (C.cal); *I. galbana* clone T-ISO and *C. calcitrans* (T-iso+C.cal) all at 20ºC and *I. galbana* clone T-ISO and *C. calcitrans* [T-iso+C.cal (22ºC)] at 22ºC.

4.3.4. Biochemical composition of broodstock

Broodstocks' showed very heterogeneous responses to the five treatments in what concerns energy storage. Variations in the contents of total lipids and glycogen (main energy reserves for gametogenesis) during the conditioning period are presented in Figure 4.3, expressed as a % of DW. The content of total lipids in the clams was significantly different among treatments [Starved ≠ (T-iso (20ºC) = C.cal (20ºC)) ≠ T-iso+C.cal (20ºC) ≠ T-iso+C.cal (22ºC)] (K–W., H=61.18, df=4, P≤0.001) and sampling times (T_0=T_8≠T_4) (K–W., H=12.09, df=2, P=0.002). In general, an increase of the percentage of total lipids was observed in clams that were fed, when compared with the initial value (T_0 – 7.22±0.96 % of DW; T_8: T-iso (20 ºC) – 8.32±1.16 % of DW; C.cal (20 ºC) – 9.86±0.35 % of DW; T-iso+C.cal (20 ºC) – 10.69±0.50 % of DW; T-iso+C.cal (22 ºC) – 10.85±0.63 % of DW). However, clams fed with T-iso+C.cal at 22 ºC increased their lipid content until the second sampling time (T_4 – 12.54±0.65 % of DW) followed by a slight decrease. In the starved broodstock a reduction in the percentage of total lipids was consistently observed during the conditioning period.

In the broodstocks' fed T-iso (20 ºC) and C.cal (20 ºC), the glycogen contents presented a slight increase from the first sampling time (T_0 – 7.50±0.64 % of DW) to the second one (T_4 – T-iso (20 ºC) – 9.35±0.61 % of DW; C.cal (20 ºC) – 8.37±0.44 % of DW; T-iso+C.cal (20 ºC) – 8.53±0.36 % of DW) with a subsequent decline until the end of the conditioning period (T_8 – Tiso (20 ºC) – 4.91±0.75 % of DW; C.cal (20 ºC) – 5.42±0.32 % of DW; T-iso+C.cal (20 ºC) –

8.38±0.16 % of DW), while the glycogen contents of the broodstock fed T-iso+C.cal at 22 ºC showed an inverse variation pattern (T-iso+C.cal (22 ºC) – T_0 – 7.50±0.64 % of DW; T_4 – 4.35±0.61 % of DW; T_8 – 8.10±0.35 % of DW). The clams fed with the diet T-iso+C.cal (20 ºC) presented a slight increase of the glycogen content during the experiment (T_0 – 7.50±0.64 % of DW; T_4 – 8.53±0.36 % of DW; T_8 – 8.38±0.16 % of DW). In the starved clams, glycogen content decreased during the conditioning period (Starved – T_0 – 7.50±0.64 % of DW; T_4 – 6.08±0.69 % of DW; T_8 – 3.57±0.41 % of DW). Significant differences were observed among treatments [(Starved = T-iso+C.cal (22ºC)) ≠ (T-iso (20ºC) = C.cal (20ºC) = T-iso+C.cal (20ºC))] (K–W., *H*=34.45, *df*=4, *P*≤0.001) and sampling times (T_0=T_4≠T_8) (K–W., *H*=13.05, *df*=2, *P*=0.001).

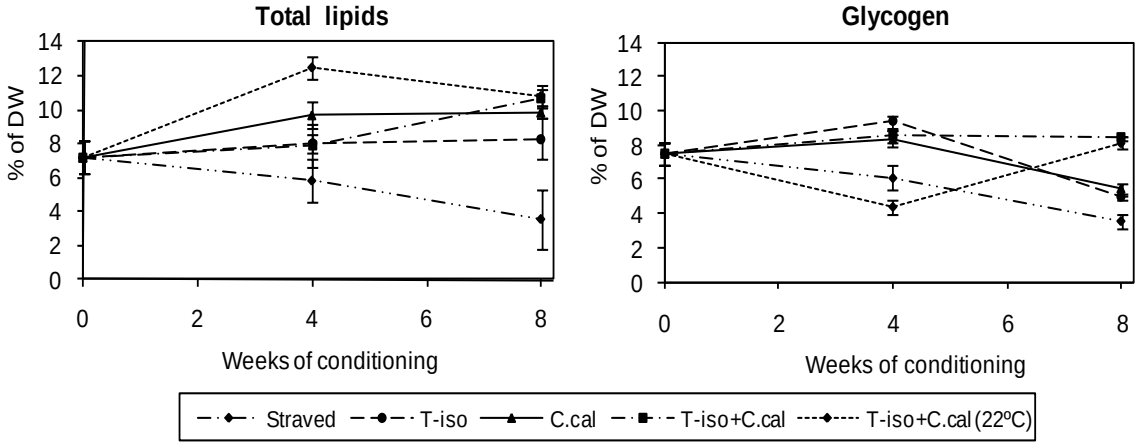

Figure 4.3. Total lipids and glycogen contents (mean±SD, *n*=5), as a percentage of dry weight, of the five broodsotck *Ruditapes decussatus* conditioned at different food regimes and temperatures: unfed (Starved); *Isochrysis galbana* clone T-ISO (T-iso); *Chaetoceros calcitrans* (C.cal); *I. galbana* clone T-ISO and *C. calcitrans* (T-iso+C.cal) all at 20ºC and *I. galbana* clone T-ISO and *C. calcitrans* [T-iso+C.cal (22ºC)] at 22ºC.

Table 4.2 presents changes in the main lipids classes of the *R. decussatus* broodstock's throughout the conditioning period under different diets and temperatures. Phospholipids, triacyglycerols and sterols constituted the more important lipid fractions of the clams in all different diets and temperatures of the conditioning.

Phospholipids ranged between 17.80 % and 53.95 % of total lipids and the proportion of phospholipids tended to decrease with sexual maturation. In fact significant differences were observed between starved and all other treatments (K–W., *H*=25.74, *df*=4, *P*≤0.001) and between initial sampling and the other sampling times (K–W., *H*=26.29, *df*=2, *P*≤0.001).

The sterol and wax esters were the least abundant lipid fraction, with quantities varying between 0 and 12.86 % of total lipids. Accumulation of this fraction in the clams tended to increase during the maturation process and was influenced by diet/temperature (K–W.,

H=14.19, *df*=4, *P*=0.007). The clams that were fed the C.cal (20 ºC) regime presented a significantly higher accumulation of sterol and wax esters than clams fed T-iso+C.cal (20 ºC) and T-iso+C.cal (22 ºC). Significant differences were observed among sampling times [$T_0 \neq (T_4 \neq T_8)$] (K–W., *H*=100.58, *df*=2, *P*≤0.001).

Sterols ranged between 17.81 % and 51.73% of total lipids and comprised 37.38 % of the total lipids in the sexually indistinguishable individuals. In general, the proportion of sterols presented a slight decrease from the first to the second sampling time with a subsequent increase until the end of the conditioning period, except for the clams conditioned with the T-iso+C.cal at 22 ºC, that revealed an overall decrease in the experimental period, and starved clams, which showed an increase during the whole conditioning period. Significant differences were observed among treatments (K–W., *H*=21.33, *df*=4, *P*≤0.001), clams in starvation presented higher values of sterols than clams fed C.cal (20 ºC) and T-iso+C.cal (20 ºC) and clams fed T-iso (20 ºC) presented higher values of this component than clams fed T-iso+C.cal (20 ºC). Significant differences were also observed among sampling times [$T_0 \neq (T_4 = T_8)$] (K–W., *H*=28.64, *df*=2, *P*≤0.001).

The free fatty acids content varied between 8.89 % and 30.65 % of clam total lipids and their evolution during the conditioning period differed according to the treatment. The clams fed with the monospecific diets [T-iso (20ºC) and C.cal (20ºC)] and T-iso+C.cal (20 ºC) presented a slight increase from the first to the second sampling time with a subsequent decrease until the end of the conditioning period, while clams in starvation and fed T-iso+C.cal at 22 ºC showed a decrease over all the experimental period. Significant differences were observed among the starved and the diets T-iso (20 ºC) e C.cal (20 ºC) (ANOVA, *F*=4.94, *df*=4, *P*<0.001). Significant differences were also observed in the sampling times [$(T_0 = T_4) \neq T_8$)] (K–W., *H*=28.64, *df* =2, *P*≤0.001).

The evolution of the triacyglycerols during the conditioning period was also clearly associated with the sexual maturation. Triacyglycerols content varied between 3.50 % and 41.50 % of total lipids. This lipid fraction had the lowest value in the sexually indistinguishable individuals at the beginning of the experiment and increased during de maturation process. Significant differences were only observed between the clams in starvation and the clams conditioned with the treatments C.cal (20 ºC) and T-iso+C.cal (22 ºC) (K–W., *H*=25.74, *df*=4, *P*≤0.001) and in the sampling times [$(T_0 \neq (T_4 = T_8)$] (K–W., *H*=58.39, *df*=2, *P*≤0.001).

Table 4.2. Composition of the lipids classes in broodstocks of *Ruditapes decussatus* throughout the conditioning period at different food regimes and temperatures: unfed (Starved); *Isochrysis galbana* clone T-ISO (T-iso); *Chaetoceros calcitrans* (C.cal); *I. galbana* clone T-ISO and *C. calcitrans* (T-iso+C.cal) all at 20ºC and *I. galbana* clone T-ISO and *C. calcitrans* [T-iso+C.cal (22ºC)] at 22ºC.

Diet	Weeks of conditioning	Phospholipids	Sterol ester+waxes	Sterols	Free fatty acids	Triacyglycerols
		% TL	% TL	% TL	% TL	% TL
Starved (20ºC)	0	38.61±11.82	nd	37.38±19.37	21.67±7.09	4.46±2.27
	4	53.95±13.12	nd	31.28±12.96	17.51±7.11	3.50±0.83
	8	53.45±37.56	2.56±1.56	51.73±18.43	8.89±7.39	3.78±2.46
T-iso (20ºC)	0	38.61±11.82	nd	37.38±19.37	21.67±7.09	4.46±2.27
	4	17.80±2.65	1.12±0.42	27.99±6.91	23.71±7.72	29.58±7.35
	8	24.67±7.96	4.26±2.86	32.31±8.35	15.22±6.53	30.79±11.87
C.cal (20ºC)	0	38.61±11.82	nd	37.38±19.37	21.67±7.09	4.46±2.27
	4	20.45±5.78	11.54±3.54	23.60±4.39	28.87±8.47	27.89±10.43
	8	23.28±7.41	12.86±3.54	26.29±10.18	18.27±5.68	32.44±17.74
T-iso+C.cal (20ºC)	0	38.61±11.82	nd	37.38±19.37	21.67±7.09	4.46±2.27
	4	30.70±8.61	nd	17.81±5.15	30.65±11.44	22.99±9.94
	8	19.21±6.46	6.60±1.83	20.40±2.81	18.66±4.25	41.50±11.15
T-iso+C.cal (22ºC)	0	38.61±11.82	nd	37.38±19.37	21.67±7.09	4.46±2.27
	4	28.97±10.99	nd	32.28±6.85	20.10±14.00	18.73±5.17
	8	20.40±6.04	11.54±2.87	21.84±10.12	19.17±5.31	38.49±9.99

The data, means of 5 five replicates, are expressed as relative percentagem of total lipids (% TL)

4.3.5. Spawning, larval rearing and oocytes biochemical composition

Spawning and veliger rate results are presented in Table 4.3. The percentage of spawners varied from 0 % to 67 %, depending on the treatment. The highest percentage of spawners was obtained with the diet T-iso+C.cal (20 ºC), followed by the diet T-iso (20 ºC). The number of females among the spawners was always lower than the males.

Table 4.3. Spawning characteristics of *Ruditapes decussatus* broodstock conditioned at different food regimes and temperatures: unfed (Starved); *Isochrysis galbana* clone T-ISO (T-iso); *Chaetoceros calcitrans* (C.cal); *I. galbana* clone T-ISO and *C. calcitrans* (T-iso+C.cal) all at 20ºC and *I. galbana* clone T-ISO and *C. calcitrans* [T-iso+C.cal (22ºC)] at 22ºC and veliger rate originating from the different treatments.

Spawning and larval parameters	Nutritional regime				
	Starved (20ºC)	T-iso (20ºC)	C.cal (20ºC)	T-iso+C.cal (20ºC)	T-iso+C.cal (22ºC)
No. of clams	40	56	52	60	56
Spawners (%)	-	54	42	67	36
Female spawners (%)	-	25	12	33	11
Mean no. eggs released (10^6)	-	0.40±0.24	0.25±0.23	0.87±0.36	0.38±0.13
D larve (%)	-	68±8	44±9	81±8	56±18

Data on the average number of eggs released by females ranged from 0.25 to 0.87 million and again it was the diet T-iso+C.cal (20 ºC) that presented the highest number, followed by the T-iso (20 ºC) diet. Significant differences were observed among the diet T-iso+C.cal (20 ºC) and the diets T-iso (20 ºC) and C.cal (20 ºC) (ANOVA, F=6.33, df=3, P=0.004). Also, the oocytes released from the females fed T-iso+C.cal (20 ºC) and T-iso (20 ºC) showed the highest percentage of the biggest oocyte classes ([63-70] and [71-78] µm) (Table 4.4). In terms of oocyte biochemical components (total lipids and carbohydrates) no significant differences were observed among treatments (ANOVA, P<0.05), however total lipids presented more differences among treatments than carbohydrates (Table 4.5).

The veliger rate was significantly higher in the treatment T-iso+C.cal (20º C) compared to the treatments C.cal (20 ºC) and T-iso+C.cal (22ºC) (K–W., H=20.98, df=3, P≤0.001). No significant differences were observed among treatments T-iso+C.cal (20 ºC) and T-iso (20 ºC) (Table 4.3).

Table 4.4. Percentage of different oocyte diameter classes (µm) from the female spawners of *Ruditapes decussatus* broodstock conditioned at different food regimes and temperatures: unfed (Starved); *Isochrysis galbana* clone T-ISO (T-iso); *Chaetoceros calcitrans* (C.cal); *I. galbana* clone T-ISO and *C. calcitrans* (T-iso+C.cal) all at 20ºC and *I. galbana* clone T-ISO and *C. calcitrans* [T-iso+C.cal (22ºC)] at 22ºC.

Oocyte diameter classes (µm)	Nutritional regime				
	Starved (20ºC)	T-iso (20ºC)	C.cal (20ºC)	T-iso+C.cal (20ºC)	T-iso+C.cal (22ºC)
47-54	-	0	9	0	0
55-62	-	8	21	2	27
63-70	-	60	47	68	52
71-78	-	32	23	30	21

The data, means of 3 replicates, are expressed as relative percentagem (%)

Table 4.5. Total lipids and carbohydrates of oocyte (mean±SD, *n*=9), from the female spawners of *Ruditapes decussatus* broodstock conditioned at different food regimes and temperatures: unfed (Starved); *Isochrysis galbana* clone T-ISO (T-iso); *Chaetoceros calcitrans* (C.cal); *I. galbana* clone T-ISO and *C. calcitrans* (T-iso+C.cal) all at 20ºC and *I. galbana* clone T-ISO and *C. calcitrans* [T-iso+C.cal (22ºC)] at 22ºC.

Biochemical composition of oocyte (ng oocyte^{-1})	Nutritional regime				
	Starved (20ºC)	T-iso (20ºC)	C.cal (20ºC)	T-iso+C.cal (20ºC)	T-iso+C.cal (22ºC)
Total lipids	-	13.93±4.67	12.96±3.44	16.16±7.45	12.34±5.10
Carbohydrates	-	6.99±1.07	7.04±1.76	7.17±1.76	6.89±1.03

4.4. Discussion

The results obtained in this study demonstrate the different nutritional values of the diets used to feed the broodstock of *R. decussatus*. The gametogenic process, energy storage and spawning success were influenced by the conditioning temperature and the nutritional value of the diet received, as evidenced by the differences in reproductive effort observed among the monospecific and combined diets.

Carbohydrates have been considered the main source of energy in bivalves (Zwann and Zandee, 1972; Barber and Blake, 1981), in particular for gametogenesis (*e.g.* Barber and Blake, 1985; Martínez, 1991). Nonetheless, Perez-Camacho et al. (2003) have showed that lipids constitute the reproductive reserve par excellence in *R. decussatus*. In fact, our results showed that the gonadal development has been more effective when the microalgae T-iso which presents a significant higher level of lipids, was part of the diet. However, the best

reproductive performance was obtained with the clams fed the combination diet of T-iso and C.cal at 20 ºC as evaluated by the greatest percentage of clams able to spawn. This result put in evidence that the European clam synthesize lipids *de novo* for gametogenesis, using stored energy reserves (carbohydrates) as substrate during vitellogenesis as previous observed in other bivalves (*e.g.* Fearman et al., 2009).

The evolution observed in the gonadal development of the individuals that were starved, suggests that in case of stress, reproduction seems to be a priority and the clams allocate all available energy to this process.

Temperature has been suggested to accelerate the reproductive conditioning of molluscs (*e.g.* Ojea et al., 2008), the present results showed indeed that the time necessary to complete gametogenesis is inversely correlated with temperature. Effectively, the gonadal development of the broodstock conditioned with the diet T-iso+C.cal at 22 ºC was accelerated with most of the females and the males attaining the reproduction period within four weeks of conditioning. However, at the end of the conditioning period, some individuals were still not in the reproduction phase, probably due to the fact that at this temperature, individuals spend more energy in the basal metabolism and therefore less energy would be allocated to somatic growth or reproduction or this might be due to the occurrence of a spontaneous spawning between week four and eight of the conditioning period. Both hypotheses could be sustained by the results of the condition index and the biochemical composition of the broodstock (total lipids and glycogen).

Beyond the gonadal development, condition index of clams has been considered as the key parameter of the sexual maturation process (*e.g.* Walne and Mann, 1975; Matias et al., 2009). The condition index of the broodstock's conditioned with the different diets is closely linked to the clam's gonadal development and the best results were observed with the clams that were fed the combination diet of T-iso and C.cal, at 20 ºC.

Several authors have reported maximum glycogen content in bivalve immediately preceding and during gamete proliferation (Ansell et al., 1980; Barber and Blake, 1985; Ojea et al., 2004). The values of glycogen and total lipid content found in the present study are in the range of the previously observed by Ojea et al. (2004) and Matias et al. (2009) for *R. decussatus*. Once again, the main energy reserves for gametogenesis (glycogen and total lipids) were directly related to the gonadal development of broodstocks. In general, glycogen tends to decrease during the maturation process while total lipids tend to increase, emphasizing the fact that *R. decussatus* during vitelogenesis utilizes the stored glycogen for *de novo* synthesis of lipids. This trend was being evident in the broodstock that was fed the combination diet of T-iso and C.cal, at 20 ºC.

The results of this study show a clear dependence on lipid content and class on *R. decussatus* broodstocks sexual maturation evolution. Not only the quantity, but also the quality

of the lipids must be considered. From a quantitative point of view of lipid classes, the importance of triacylglycerols was greater than the other classes, this group showed a clear relationship with gonadal development. In the sexually indistinguishable individuals at the beginning of the experiment, tryacylglycerols represented only 4 % of the total lipids, but with the sexual development the accumulation of triacylglycerols was evident. Napolitano et al. (1992) have established for the species *Placopecten magellanicus* that triacylglycerols and acylglycerides were the principal constituents of oocytes, whose content was clearly related to the condition index of the feminine gonad and the maximum oocyte diameter. Although, no significant differences were observed between the broodstock fed the diet T-iso+C.cal at 20 ºC and the other treatments, this broodstock showed the higher level of the triacylglycerols and the maximum oocyte diameter. This is the first evidence in *R. decussatus*.

Our results put in evidence the quantitative importance of phospholipids in *R. decussatus*, confirming the previously description by Beninger and Lucas (1984), Delgado et al. (2004) and Fernández-Reiriz et al. (1999). Phospholipids have a clear structural function and are a fundamental part of the cellular membranes, having a role in the formation of oocytes and spermatozoids, and in the general growth of the organism. Moreover, phospholipids function as energetic reserves in the eggs of *R. decussatus* (Benninger and Lucas, 1984; Delgado et al., 2004; Matias et al., 2011).

Sterols and free fatty acids, showed no clear sexual maturation trend.

Reproductive output is defined by three major components: spawning success, oocyte number and diameter (Pronker et al., 2008). In this study spawning success and fecundity varied between the five broodstock groups as a result of the different in diet and temperature regimes. Animals that received the T-iso+C.cal diet at 20 ºC had a much higher spawning success, with 67 % of the females spawning, releasing an average of $0.87*10^6$ eggs per female, indicating that this diet and temperature was the most adequate for providing energy for gamete production. Nutritional stress reduces the spawning success and fecundity in proportion to the decline of energy available for gamete production (Pronker et al., 2008). The different fecundity observed among the diets can be attributed to the influence of the different biochemical composition of the microalgae (T-iso presented a significantly higher level of lipids). Besides the gross composition of microalgae, many other factors could contribute to their nutritional value, for example vitamins, minerals, and pigments (Brown, 2002).

We observed a decrease in oocyte numbers as well as a decrease in oocyte size under unfavourable conditions. The minimum oocyte class diameter [47-54 µm] was observed in the diet C.cal (20 ºC). This treatment also shows the lowest veliger rate, indicating that this oocyte diameter class might be the lower limit for producing viable oocytes. In contrast, the diet T-iso+C.cal at 20 ºC presented the highest number of oocytes released, the highest percentage of the biggest oocyte classes [71-78 µm] and the higher veliger rate. Previous studies showed

that indeed oocytes produced under nutritional stress are smaller and contain less organic matter than eggs produced under optimal conditions (Pronker et al., 2008). If there is a minimum oocyte size under which it is not profitable to produce oocyte at all, oocyte numbers should decrease more than oocyte size under unfavourable conditions (Hendriks et al., 2003). Our results sustain for the first time that this theory can be applied to *R. decussatus*.

In term of oocyte biochemical components (total lipids and carbohydrates), the relation between the biochemical contents of the broodstocks and the oocytes indicate that the parental total lipids was related with the oocyte lipids, however this was not evident for the carbohydrates.

The first priority for the assimilated energy in clams is basal metabolism, and any exceeding energy is then allocated to somatic growth (tissue and/or shell), store glycogen, or reproduction, with stored glycogen accessible as an energy source for reproductive maturation (Fearman et al., 2009). A diet providing a combination of both essential lipids and simple carbohydrates leads clams to allocate energy to reproduction, and simultaneously to storage or to the increase in the energetic demands of reproductive maturation. It is clear that gametogenesis requires a supply of essential lipids from the diet (namely triacylglycerols), along with sufficient energy reserves (carbohydrates). At the end of this experiment the combination diet at 20 ºC, produced the highest percentage of clams that were able to spawn, the higher spawning success, the highest fecundity and the highest veliger rate.

Two main conclusions emerge from the present study: i) temperature is a parameter which must be carefully managed to improve the reproductive conditioning of bivalves, although the highest temperature throughout gametogenesis shortened the time to full ripeness, if did not produce a better reproductive output; ii) lipids are clearly of major importance in conditioning, either as energy reserves or as precursors of tissue structures being triacylglycerols the ones which play the more important role. This study also demonstrate that the combination diet (T-iso + C.cal) at 20 ºC used is the highly suitable to provide maximal reproductive effort and output performance of *R. decussatus* broodstock conditioning. This study can also contribute to improve global hatchery technological development of this emerging species, mainly concerning the broodstock conditioning and consequently production of high quality spat.

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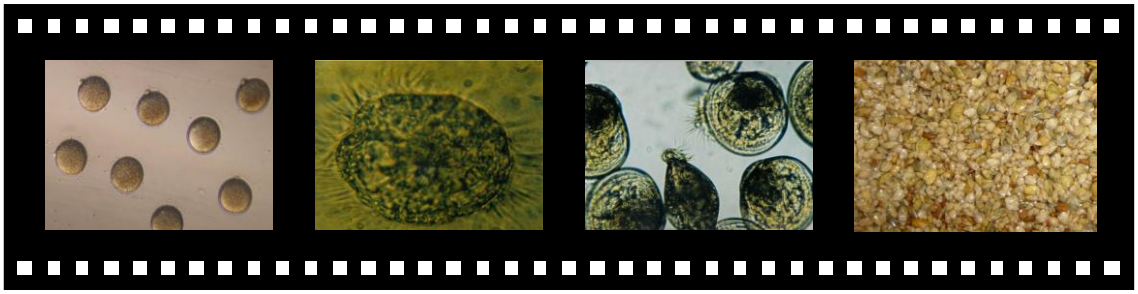
Chapter 5

Biochemical compounds dynamics during larval development of the carpet-shell clam *Ruditapes decussatus* (Linnaeus, 1758): Effects of monospecific diets and starvation

Matias D., Joaquim S., Ramos M., Sobral P., Leitão A., 2011. Biochemical compounds' dynamics during larval development of the carpet-shell clam *Ruditapes decussatus* (Linnaeus,1758): effects of monospecific diets and starvation. Helgoland Marine Research 65(3):369-380.



Abstract

Successful larval growth and development of bivalves depend on energy derived from internal (endotrophic phase) and external (exotrophic phase) sources. The present paper studies survival, growth and biochemical changes in the early developmental stages (from egg to pediveliger) of the clam *Ruditapes decussatus* in order to characterize the nutritional requirements and the transition from the endotrophic to the exotrophic phase. Three different feeding regimes were applied: starvation, and two monospecific microalgal diets (*Isochysis aff galbana* and *Chaetoceros calcitrans*). A comparison between fed and unfed larvae highlighted the importance of egg lipid reserves, especially neutral lipids, during a brief endotrophic phase of embryonic development (first 2 days after fertilization). Egg reserves, however, may energetically contribute to the maintenance of larvae beyond the embryonic development. In fed larvae, the endotrophic phase is followed by a mixotrophic phase extending to days 5-8 after fertilization, and a subsequent exotrophic phase. Metamorphosis starts around day 20. The intense embryonic activities are supported by energy derived from lipids, mainly from neutral lipids, and the metamorphic activities are supported by energy derived essentially from proteins accumulated during the planktonic phase and depend on the nutritional value of diets. The diet of *I. aff galbana* proves to be more adequate to *R. decussatus* larvae rearing. The results provide useful information for a successful production of *R. decussatus* aquaculture.

Keywords: Bivalve larvae; *Ruditapes decussatus*; Biochemical composition; Nutritional phases; Starvation; Diet.

5.1. Introduction

The clam *Ruditapes decussatus* is widely distributed along the coastal and estuarine areas of Europe and North Africa and has high commercial value. Nevertheless, information on its biology and especially its larval phase is very limited (Chícharo and Chícharo, 2001; Beiras and Albentosa, 2004).

The major morphological changes in bivalve development occur during embryogenesis and metamorphosis. Successful bivalve larval growth and development depend on the energy available during the endotrophic and the subsequent exotrophic developmental phases (Labarta et al., 1999b; Pernet et al., 2004). The endotrophic phase largely corresponds to the embryonic development and is mainly managed by endogenous reserves provided to the eggs during oogenesis (Bayne, 1973). The subsequent exotrophic phase, which leads to larval metamorphosis, depends on the value of the diet provided to promote larval growth (Whyte et al., 1990). Both phases are characterized by intense morphogenetic activities, while there is only a small increase in size (Bayne et al., 1975). The transition from the endotrophic to the exotrophic phase seems to be gradual; larvae initiate feeding while still using their yolk reserves. There are only a few published studies on this transitional phase (Lucas et al., 1986; Delaunay, 1992; Rico-Villa et al., 2009).

The roles of tissue proteins, lipids and carbohydrates differ among bivalve species. The viability of bivalve larvae is limited by the accumulation and/or utilization of either lipids (Millar and Scott, 1967; Helm et al., 1973; Holland and Spencer, 1973) or proteins (Bartlett, 1979; Rodríguez et al., 1990), but not carbohydrates (Collyer, 1957; Holland and Spencer, 1973; Bartlett, 1979; Gallager and Mann, 1986; Gallager et al., 1986; Labarta et al., 1999a), although Haws et al. (1993) suggested that carbohydrates may play a part in the optimal utilization of other reserves.

The importance of lipids as an energy reserve in pelagic veliger larvae and the relationship between maximal viability and survival of cultured larvae and the initial lipid (especially neutral lipids) content have been reported (Helm et al., 1973; Holland, 1978; Gallager et al., 1986; Ferreiro et al., 1990). There is also evidence that lipids accumulated during the pelagic larval phase are used as the principal energy substrates during metamorphosis in several bivalve species (Holland and Spencer, 1973; Gabbott, 1976). In contrast, Bartlett (1979) demonstrated that *Crassostrea gigas* accumulates more energy in the form of proteins than neutral lipids, suggesting that in this species protein is the basic energy reserve during metamorphosis. Similar observations were made by Rodríguez et al. (1990) for *Ostrea edulis*.

In the present study we compare fed and unfed *R. decussatus* larvae in order to evaluate the development and duration of the endotrophic/exotrophic transition period and to understand the selective use of the different biochemical substrates in both absolute and energetic terms during larval development. A better understanding of the species' energy metabolism and

nutritional requirements would be relevant to a successful production of *R. decussatus* in aquaculture.

5.2. Materials and Methods

5.2.1. Microalgae culture conditions

The microalgae *Isochrysis aff galbana* (T-iso) and *Chaetoceros calcitrans* (C.cal) were reared in 10 l flasks with f/2 medium (Guillard, 1975), in a temperature-controlled room at 20±2 ºC under continuous illumination. Seawater (salinity = 36±1) was filtered (0.45 µm) and UV-treated. A continuous aeration was provided to enhance growth and prevent the algae from settling. Algae were harvested when the culture had reached the stationary growth phase. Before being used as food, algal cells were counted with a Coulter Counter TA II, 100 µm, from aliquots of 0.5 ml from each culture.

5.2.2. Broodstock conditioning

Adult *R. decussatus* (>35 mm shell length) were collected in March 2006 from Ria de Aveiro (40º42′N; 08º40′W) (western coast of Portugal). Broodstock clams were conditioned at 20±1 ºC for 2 months to speed up their gonad development. Clams were continuously fed a mixture of T-iso and C.cal ($5x10^8$cells $ind^{-1}d^{-1}$) in a 1:1 ratio in terms of size, the dry weight of algae corresponding to 4 % of the dry weight of the clams' soft tissue (Utting and Millican, 1997). The water was enriched with this mixed diet and distributed to the tanks at a flow rate of 0.6 to 0.8 l min^{-1}. Clams were induced to spawn by a rapid increase of temperature from 20 ºC to 28±1 ºC over a 6 h interval.

5.2.3. Experimental design

Oocytes from all females spawned (*n*=5) were pooled and mixed with sperm (about ten spermatozoids per oocyte) for fertilization. Fertilized eggs were collected on a sieve, washed with filtered seawater and redistributed in a known volume of filtered seawater, subsampled and counted. Three samples of about 50,000 eggs each were taken for biochemical analyses, and a sample of 50 eggs was also taken for diameter measurement. The fertilized eggs were incubated in triplicate 5 l tanks, with filtered UV irradiated seawater, maintained at 22±1 ºC, at a density of 100 eggs per ml with slight aeration. After 48 h, the D-larvae were collected on a 30 µm mesh screen and the total number of veligers was calculated based on two 1 ml aliquots. The early D-larvae collected were dispensed at an initial density of 10±2 larvae ml^{-1} in 5 l tanks with natural

filtered seawater (0.45 µm) and reared in triplicate under three nutritional regimes: unfed and fed with monospecific algae (T-iso and C.cal, respectively). Food was added daily to each tank at a rate of 50 cells μl_{-1}. Salinity was 36±1 and temperature was maintained at 22±1 ºC. Water was renewed every 2-3 days. At each time, samples were taken from each tank in order to estimate survival and mean shell length and to detect presence of foot and/or any morphological alterations in the velum. Samples of 5,000 larvae each were also taken for biochemical analyses on days 2, 5, 8, 14, 19 and 23. Remaining larvae were transferred to the tanks.

Percent larval survival was determined at each water renewal. Antero-posterior shell length was measured for 50 randomly sampled larvae from each replicate using an ocular micrometer. Linear regressions were fitted to length over larval growth trajectories to determine length growth rates from eggs to metamorphic larvae for each treatment.

The presence of a foot was scored to determine larval development status. Larvae that showed a clearly visible foot bulging out of the shell (pediveligers) was considered metamorphosed.

5.2.4. Biochemical composition of eggs and larvae

Samples of eggs and larvae for biochemical analyses were rinsed with iso-osmotic ammonium formate (3 % w/v) to remove salt, transferred to Eppendorf tubes, frozen and stored in liquid nitrogen and then freeze-dried. A micro-analytical fractionated extraction scheme developed by Holland and Gabbott (1971) and Holland and Hannant (1973) was followed for the determination of the contents of biochemical components. Lyophilized samples were homogenized in 500 µl distilled water using a sonicator. Samples were sonicated in an ice water bath for three intervals of 10 s at 20 W each to obtain a thoroughly homogenized sample. Separate samples (200 µl) of the initial homogenate were taken for analyses of total lipids, neutral lipids, proteins, total carbohydrates and free reducing sugars. Total lipid content was extracted by the method of Bligh and Dyer (1959) and taken up in 500 µl chloroform.

Total lipids were determined by the methods of Marsh and Weinstein (1966) using tripalmitin as a standard, and the absorbance was determined at 375 nm. Neutral lipids were determined in the same way as total lipids; 200 µl samples of neutral lipids in chloroform were dried for 20 min at 100 ºC and used for determinations. Phospholipids were determined as the difference between total and neutral lipids.

Proteins were precipitated by cold 5 % trichloroacetic acid (TCA) and the precipitate washed in warm 1.0 N NaOH. Protein concentration was assayed by the method of Lowry at al. (1951), modified by Bensadoun and Weinstein (1976) and Hess et al. (1978), at 750 nm using serum albumin as a standard.

Hydrolysed and unhydrolysed samples of TCA supernatant were used for the determination of total carbohydrates and free reducing sugars by a modification of the method of Folin and Malmros (1929). The components were quantified with a ferricyanate reduction reaction at 420 nm using glucose as a standard. Polysaccharides were determined as the difference between carbohydrates and free reducing sugars.

The organic matter was the sum of proteins, total lipids and carbohydrates. Linear regressions were fitted to organic matter over larval growth trajectories to determine organic growth rates from eggs to metamorphic larvae for each nutritional condition.

Energy conversion factors used for lipid, carbohydrate and protein were 35.24, 17.16 and 18.00 KJ g^{-1}, respectively (Beukema and De Bruin, 1979).

5.2.5. Statistical analyses

Differences in survival, growth (shell length, shell length growth rate, organic matter and organic matter growth rate), biochemical composition and energy were tested by analyses of variance (ANOVA) or Kruskal–Wallis ANOVA on ranks, whenever the assumptions of ANOVA failed among days of the same nutritional regime and between regimes. Multiple pair comparisons among means were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test. Percentage data were arcsine transformed to normalize variance (Sokal and Rohlf 1981). Results were considered significant at $P<0.05$. The statistical analyses were undertaken using the SIGMASTAT 3.11 statistical package.

5.3. Results

5.3.1. Survival and growth

Survivorship of starved larvae was high till day 5 after fertilization (96.27±1.68 %), but then showed a steep decline (Figure 5.1). On day 23 (last record), 23.21±3.95 % of the larvae (equivalent to 13,000±2,677 larvae) were still active. In contrast, the survival of fed larvae showed a rather gradual decrease over the experimental period. This decrease was slightly more pronounced after day 14, when metamorphosis began. Highest survival rates were observed in larvae fed with C.cal (71.98±12.25 %), although these values were not significantly different from those of larvae fed with T-iso (67.00±13.70 %). Significant differences in survival rates were, however, observed between unfed and fed larvae (K–W., $H=12.77$, $df=2$, $P=0.002$).

The results for growth (length and organic matter) and the presence of foot in clam larvae under the three nutritional regimes are detailed in Table 5.1. The diameter and organic matter of

eggs (day 0) as well as the initial length and organic matter of the early D-veliger larvae (day 2) were the same for all three treatments. During the early developmental stages (from egg to early D-veliger larva), organic matter remained almost constant (27.48±3.55 ng ind^{-1} to 29.59±1.65 ng ind^{-1}).

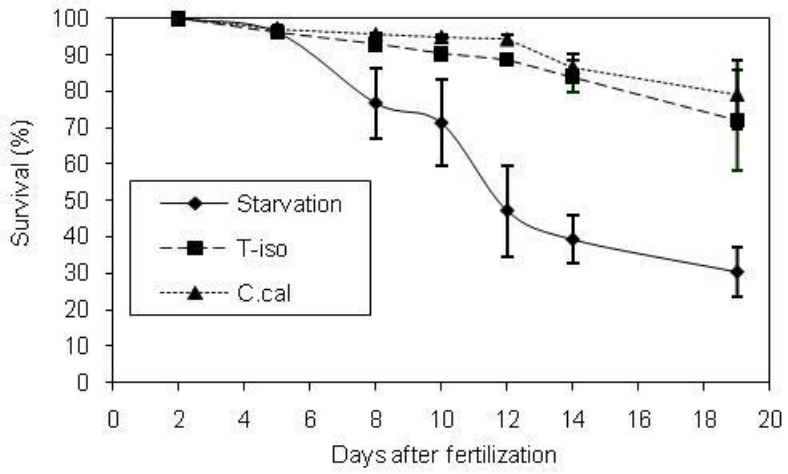


Figure 5.1. Survival (mean ± SD, *n*=6) of *Ruditapes decussatus* larvae reared under three levels of nutrition: starvation and two monospecific diets (*Isochysis aff galbana* = T-iso, and *Chaetoceros calcitrans* = C.cal).

The growth rate of unfed larvae was only 0.33 µm day^{-1}, but significant differences in larvae growth were found among days after fertilization (K–W., *H*=82.45, *df*=7, *P*<0.001). In terms of organic matter a sudden decrease was observed especially following day 5 after fertilization. When expressed as change in organic matter, negative values for growth rates were obtained (-0.91±0.02 ng ind^{-1}), indicating a progressive decrease in the organic tissue of unfed larvae.

In contrast, larvae reared with the monospecific diets showed an increase in length and also in organic matter. Larvae fed with C.cal had the highest length growth rate (4.95±0.02 µm day^{-1}), while larvae fed with T-iso showed the highest organic matter growth rate (16.14±1.30 ng day^{-1}). The percentage of foot presence at day 23 was higher in larvae fed with T-iso (75.66 %) than in those fed with C.cal (42.81 %). Significant differences among the three nutritional regimes were observed in length (K–W., *H*=908.03, *df*=2, *P*≤0.001), length growth rate (ANOVA, *F*=2533.93, *df*=2, *P*<0.001) and organic matter growth rate (ANOVA, *F*=365.81, *df*=2, *P*<0.001). With respect to organic matter, however, significant differences were found only between fed and unfed larvae (K–W., *H*=34.36, *df*=2, *P*≤0.001).

Table 5.1. Length (egg diameter and shell length, respectively; mean±SD, *n*=150), larval length growth rate (mean±SD, *n*=3), organic matter (mean±SD, *n*=3), organic matter growth rate (mean±SD, *n*=3), and presence of foot (%) during the early development of the clam *Ruditapes decussatus* under three levels of nutrition: starvation and two monospecific diets (*Isochysis aff galbana* = T-iso and *Chaetoceros calcitrans* = C.cal).

Food regime	Days after fertilization	Lenght (µm ind^{-1})	Larval length growth rate (µm day^{-1})	Organic matter (ng ind^{-1})	Organic matter growth rate (ng day^{-1})	Foot (%)
Starvation	0	58.10±4.18		27.48±3.55		
	2	96.71±5.98		29.59±1.65		
	5	100.18±5.70		35.38±4.22		
	8	99.18±4.30		26.59±5.35		
	10	101.36±5.60		-		
	12	102.31±4.45		-		
	14	102.27±6.05		14.59±1.02		
	19	104.53±6.64		10.38±1.71		
	23	103.75±13.70	0.33±0.09	8.16±0.63	-0.91±0.02	0.00
T-iso	0	58.10±4.18		27.48±3.55		
	2	96.71±5.98		29.59±1.65		
	5	103.53±5.02		113.19±12.95		
	8	112.48±4.69		199.19±15.24		
	10	119.21±7.02		-		
	12	129.81±8.66		-		
	14	150.14±10.10		232.78±26.24		
	19	179.25±9.72		431.84±71.31		
	23	184.03±9.32	4.69±0.12	322.17±7.56	16.14±1.30	75.66
C.cal	0	58.10±4.18		27.48±3.55		
	2	96.71±5.98		29.69±2.03		
	5	104.27±6.50		80.65±6.11		
	8	134.81±15.30		96.65±2.93		
	10	153.88±6.46		-		
	12	161.53±9.99		-		
	14	168.48±11.64		149.98±11.83		
	19	174.39±13.36		180.98±15.82		
	23	199.67±12.19	4.95±0.02	143.20±7.78	6.31±0.31	42.81

5.3.2. *Biochemical composition and energy contents*

Data on the biochemical composition (proteins, total lipids and carbohydrates) of eggs and larvae are presented in Table 5.2. Proteins and total lipids were the major biochemical components of early stages of *R. decussatus*. Proteins and total lipids remained almost constant until the early D-veliger stage (13.98±3.39 to 14.77±0.57 ng ind^{-1}, and 8.10±2.40 to 7.34±0.00 ng ind^{-1}, respectively), while carbohydrates nearly duplicated (2.31±0.92 to 4.14±0.14 ng ind^{-1}) during this period. Generally, after embryogenesis and trochophore development (day 2 after fertilization) the above biochemical components tended to increase in fed veligers till day 19, but decreased between days 19 and 23. Larvae fed with T-iso presented the highest increase in all three

biochemical components. Protein contents increased from 14.77±0.57 to 144.33±11.65 ng ind^{-1} (T-iso) and 72.35±5.08 ng ind^{-1} (C.cal), and total lipids from 7.34±0.00 to 118.09±8.27 ng ind^{-1} (T-iso) and 47.22±4.99 ng ind^{-1} (C.cal). Carbohydrates showed the same tendency: in larvae fed with T-iso their contents increased from 4.14±0.14 to 38.29±4.56 ng ind^{-1}, while larvae fed with C.cal showed irregular variations in carbohydrate contents after day 8.

Table 5.2. Principal biochemical composition (mean±SD, *n*=9) of early developmental stages of the clam *Ruditapes decussatus* under three levels of nutrition: starvation and two monospecific diets (*Isochysis aff galbana* = T-iso and *Chaetoceros calcitrans* = C.cal).

Food regime	Days after fertilization	Gross biochemical composition (ng ind^{-1})		
		Proteins	Total lipids	Carbohydrates
Starvation	0	13.98±3.39	8.10±2.40	2.31±0.92
	2	14.77±0.57	7.34±0.00	4.14±0.14
	5	20.22±3.92	7.24±0.83	4.36±0.41
	8	13.49±6.84	5.58±0.83	4.29±0.47
	14	4.75±1.28	2.82±1.26	4.03±0.40
	19	2.39±1.26	1.72±0.48	3.51±0.20
	23	0.37±0.00	1.72±0.48	3.31±0.29
T-iso	0	13.98±3.39	8.10±2.40	2.31±0.92
	2	14.37±0.00	7.34±0.00	4.14±0.14
	5	54.86±14.13	25.16±1.91	16.56±0.92
	8	84.46±11.48	61.28±2.66	34.64±1.03
	14	101.28±26.23	72.59±4.14	39.01±1.73
	19	212.94±55.97	151.18±30.41	46.26±9.47
	23	144.33±11.65	118.09±8.27	38.29±4.56
C.cal	0	13.98±3.39	8.10±2.40	2.31±0.92
	2	14.37±0.00	7.34±0.00	4.14±0.14
	5	36.70±2.02	21.58±4.08	19.56±0.85
	8	27.96±3.08	46.39±1.72	20.41±1.13
	14	68.99±5.34	53.01±9.11	18.59±1.03
	19	85.81±13.44	56.04±2.48	32.55±1.16
	23	72.35±5.08	47.22±4.99	23.78±6.21

In the unfed culture (see also Figure 5.2), the protein contents showed a slight initial increase between days 2 and 5, followed by an abrupt decrease to a minimum value of 0.37±0.00 ng ind^{-1} on day 23, which represents a decrease of 27.81 % in terms of total organic matter. A similar situation was observed for total lipids; the level remained constant till day 5 followed by a gradual decline till the end of the experiment (day 2: 7.34±0.00 ng ind^{-1}; day 23: 1.72±0.48 ng ind^{-1}), which represents a decrease of 12.94 % in total organic matter. No significant changes were observed in proteins (K-W., *H*=13.12, *df*=4, *P*=0.011, Dunn's Method, *P*>0.05) and total lipids

(ANOVA, *F*=26.93, *df*=4, P<0.001, Tukey Test, *P*>0.05) during the first 8 days after fertilization. Both components presented minimum values at the end of the experimental period (day 23). In contrast, the carbohydrate contents remained nearly constant throughout the experimental period. Significant differences were observed in biochemical composition (proteins - K–W., *H*=32.99, *df*=2, *P*≤0.001; total lipids - K–W., *H*=34.03, *df*=2, *P*≤0.001 and carbohydrates - K–W., *H*=30.63, *df*=2, *P*≤0.001) between unfed and fed larvae.

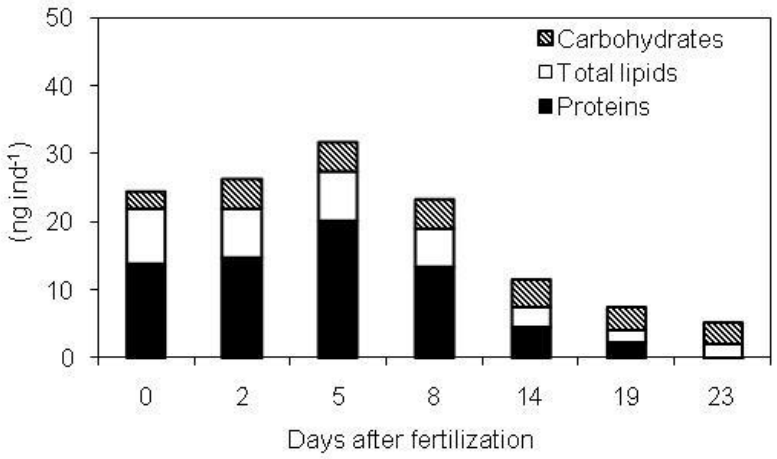


Figure 5.2. Biochemical composition (mean, *n*=9) of early developmental stages of the clam *Ruditapes decussatus* reared without food.

The results for neutral lipids, phospholipids, free reducing sugars and polysaccharides in eggs and larvae under the three feeding regimes are shown in Table 5.3. The main contribution to total lipids came from neutral lipids. During the development from eggs to early D-veliger larvae, a slight reduction in energetic lipids (neutral lipids) was observed (from 7.30±2.43 to 5.89±0.00 ng ind^{-1}) while structural lipids (phospholipids) increased (from 0.08±0.04 to 1.45±0.00 ng ind^{-1}) for all three treatments. The veliger larvae fed with T-iso showed the greatest increase in both neutral lipids and phospholipids. Neutral lipids progressively increased till day 19 (106.77±9.21 ng ind^{-1}) with a subsequent decrease till day 23 (60.04±29.46 ng ind^{-1}). Phospholipid contents increased till day 23 when 58.04±27.34 ng ind^{-1} was attained. In larvae fed with C.cal, neutral lipids showed lower values (day 23: 23.78±6.21 ng ind^{-1}) but the same tendency as in larvae fed T-iso. A rapid increase in phospholipid contents from day 5 to day 8 was followed by a decrease between days 8 and 14, while the contents remained constant between days 14 and 23. In unfed larvae the neutral lipid contents showed a significant decrease from day 5 to day 14 (ANOVA, *F*=33.16, *df*=4, *P*<0.001), *i.e.*, there was a rapid utilization of energetic lipids reserves, while the phospholipid contents did not vary significantly throughout the experiment (ANOVA, *F*=0.58, *df*=4, *P*=0.683) (Table 5.3 and Figure 5.3). These findings give a clear idea of the pattern of neutral lipids utilization (13.18 % of organic matter) during the early development of *R. decussatus*. Significant differences were observed in neutral lipids (K–W., *H*=29.90, *df*=2, *P*≤0.001) and phospholipids (K–W.,

H=27.58, *df*=2, *P*≤0.001) between unfed and fed larvae. Free reducing sugars and polysaccharides increased in all treatments during development from eggs to early D-veliger larvae. From the earliest shell stage to the pediveliger stage, the free reducing sugar levels in fed larvae increased from 3.35±0.14 to 40.58±9.90 ng ind^{-1} (day 19) with a subsequent decrease till day 23 (26.74±3.65 ng ind^{-1}) in larvae fed with T-iso. Larvae fed with C.cal presented the same trend, with the highest value recorded on day 19 (27.17±11.02 ng ind^{-1}). The polysaccharide contents of fed larvae increased until day 8 and then decreased till the end of experiment, except for the final measurement (day 23) in T-iso fed larvae. In the unfed culture, free reducing sugars remained almost constant over the experimental period and the same tendency was observed for the polysaccharide contents, although a slight decrease was detected following day 14. Significant differences were observed in free reducing sugars (K–W., *H*=31.31, *df*=2, *P*≤0.001) and polysaccharides (K–W., *H*=29.42, *df*=2, *P*<0.001) between unfed and fed larvae.

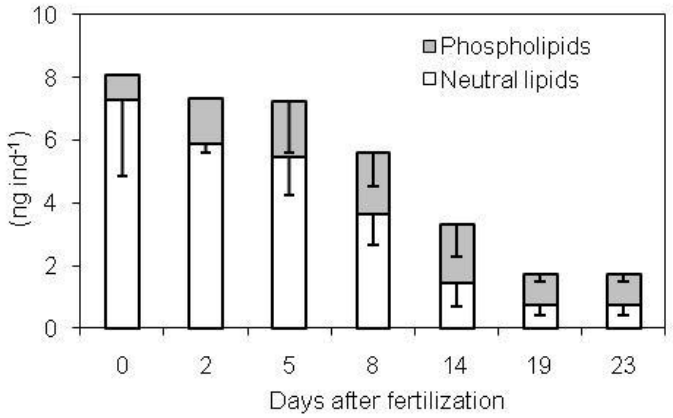


Figure 5.3. Neutral lipids and phospholipids (mean±SD, *n*=9) in early developmental stages of the calm *Ruditapes decussatus* reared without food.

Table 5.3. Neutral lipids, phospholipids, free reducing sugars and polysaccharides (mean±SD, *n*=9) in early developmental stages of the clam *Ruditapes decussatus* under three levels of nutrition: starvation and two monospecific diets (*Isochysis aff galbana* = T-iso and *Chaetoceros calcitrans* = C.cal).

Food regime	Days after fertilization	Biochemical composition (ng ind^{-1})			
		Neutral lipids	Phospholipids	Free reducing sugars	Polysaccharides
Starvation	0	7.30±2.43	0.08±0.04	1.76±0.98	0.56±0.15
	2	5.89±0.00	1.45±0.00	3.35±0.14	0.78±0.00
	5	5.44±1.22	1.79±1.72	3.71±0.16	0.65±0.32
	8	3.65±1.00	1.93±1.04	3.64±0.32	0.65±0.20
	14	1.45±0.74	1.86±1.04	3.31±0.16	0.72±0.29
	19	0.76±0.34	0.97±0.24	3.05±0.16	0.46±0.16
	23	0.76±0.34	0.97±0.24	2.99±0.20	0.32±0.17
T-iso	0	7.30±2.43	0.08±0.04	1.76±0.98	0.56±0.15
	2	5.89±0.29	1.45±0.00	3.35±0.14	0.78±0.00
	5	16.20±1.84	8.96±1.57	11.60±0.78	4.96±0.92
	8	44.60±1.77	16.68±3.34	24.26±0.85	10.38±0.54
	14	44.98±13.30	26.61±18.19	33.01±0.88	6.00±1.86
	19	106.09±9.21	45.09±23.55	40.58±9.90	5.68±2.49
	23	60.04±29.46	58.04±27.34	26.74±3.65	11.32±4.58
C.cal	0	7.30±2.43	0.08±0.04	1.76±0.98	0.56±0.15
	2	5.89±0.00	1.45±0.00	3.35±0.14	0.78±0.00
	5	16.06±1.63	5.52±4.82	8.08±0.82	11.49±1.39
	8	18.26±8.12	31.50±13.41	9.84±0.69	10.57±1.53
	14	29.57±5.13	23.44±9.31	13.36±1.04	5.22±1.48
	19	30.81±4.56	23.99±3.51	27.17±11.02	5.38±1.51
	23	23.78±6.21	23.44±8.91	10.49±1.15	4.05±0.99

Table 5.4. Energy equivalents of the principal biochemical component (mean±SD, *n*=9) of the early developmental stages of the clam *Ruditapes decussatus* reared under three levels of nutrition: starvation and two monospecific diets (*Isochysis aff galbana* = T-iso, and *Chaetoceros calcitrans* = C.cal).

Food regime	Days after fertilization	Energy content (KJ ind^{-1})			
		Proteins	Total lipids	Carbohydrates	Total energy
Starvation	0	0.25±0.06	0.29±0.08	0.04±0.02	0.58±0.07
	2	0.26±0.00	0.26±0.00	0.10±0.00	0.62±0.00
	5	0.36±0.06	0.26±0.03	0.09±0.02	0.71±0.07
	8	0.24±0.10	0.19±0.03	0.06±0.01	0.50±0.07
	14	0.09±0.03	0.10±0.04	0.03±0.01	0.21±0.07
	19	0.04±0.02	0.06±0.02	0.01±0.00	0.12±0.03
	23	0.01±0.00	0.06±0.02	0.01±0.00	0.08±0.02
T-iso	0	0.25±0.06	0.29±0.08	0.04±0.02	0.58±0.07
	2	0.26±0.00	0.26±0.00	0.10±0.00	0.62±0.00
	5	0.99±0.25	0.88±0.07	0.28±0.01	2.16±0.20
	8	1.52±0.21	2.16±0.09	0.59±0.01	4.27±0.30
	14	1.82±0.47	2.56±0.15	0.67±0.01	5.05±0.42
	19	3.83±1.01	5.32±1.07	0.79±0.16	9.95±1.88
	23	2.60±0.21	4.16±0.29	0.66±0.08	7.42±0.23
C.cal	0	0.25±0.06	0.29±0.08	0.04±0.02	0.58±0.07
	2	0.26±0.00	0.26±0.00	0.10±0.00	0.62±0.00
	5	0.66±0.04	0.76±0.14	0.34±0.01	1.76±0.16
	8	0.50±0.06	1.63±0.06	0.35±0.01	2.49±0.03
	14	1.24±0.10	1.87±0.32	0.32±0.02	3.43±0.36
	19	1.41±0.03	2.02±0.06	0.56±0.20	3.98±0.29
	23	1.30±0.09	1.66±0.18	0.25±0.02	3.21±0.22

Table 5.5. Energy differentials during larval development of the clam *Ruditapes decussatus* reared under three levels of nutrition: starvation and two monospecific diets (*Isochysis aff galbana* = T-iso, and *Chaetoceros calcitrans* = C.cal).

Food regime	Days after fertilization	Energy differentials (KJ ind^{-1})			
		Proteins	Total lipids	Carbohydrates	Total energetic
Starvation	0-2	0.01±0.00	**0.03±0.00**	0.06±0.00	0.04±0.00
	2-5	0.11±0.03	**0.00±0.03**	**0.01±0.02**	0.09±0.07
	5-8	**0.12±0.11**	**0.06±0.05**	**0.03±0.00**	**0.21±0.10**
	8-14	**0.16±0.13**	**0.10±0.06**	**0.04±0.01**	**0.29±0.09**
	14-19	**0.04±0.03**	**0.04±0.04**	**0.01±0.00**	**0.09±0.06**
	19-23	**0.04±0.02**	0.00±0.03	0.00±0.01	**0.04±0.05**
T-iso	0-2	0.01±0.00	**0.03±0.00**	0.06±0.00	0.04±0.00
	2-5	0.73±0.25	0.63±0.07	0.21±0.01	1.57±0.20
	5-8	0.53±0.46	1.27±0.09	0.31±0.01	2.12±0.50
	8-14	0.30±0.46	0.40±0.07	0.08±0.01	0.78±0.41
	14-19	2.01±1.23	2.77±0.97	0.12±0.16	4.90±1.82
	19-23	**1.24±1.08**	**1.17±0.79**	**0.14±0.24**	**2.54±1.65**
C.cal	0-2	0.01±0.00	**0.03±0.00**	0.06±0.00	0.04±0.00
	2-5	0.40±0.04	0.50±0.14	0.26±0.01	1.17±0.16
	5-8	**0.16±0.08**	0.87±0.11	0.01±0.01	0.73±0.18
	8-14	0.74±0.15	0.23±0.30	**0.03±0.02**	0.94±0.33
	14-19	0.20±0.13	0.07±0.47	0.24±0.19	0.50±0.79
	19-23	**0.11±0.10**	**0.44±0.08**	**0.31±0.23**	**0.85±0.04**

The increments represent the difference with the preceding stage.

The values in bold type are negative.

Table 5.4 shows the energy content generated by each biochemical component analyzed and the total energy (KJ ind^{-1}). The main contributions to the total energy came from proteins and total lipids. The energy generated by proteins and total lipids was approximately 40 % and 50 % of total energy, respectively whereas carbohydrates contributed only with about 10 %. During larval development significant differences were observed between unfed and fed larvae in terms of total energy (K–W., H=28.95, df=2, P≤0.001) and energy generated by proteins (K–W., H=28.25, df=2, P≤0.001), total lipids (K–W., H=33.28, df=2, P≤0.001) and carbohydrates (K–W., H=23.67, df=2, P≤0.001). In starved larvae a gradual decrease in total energy and energy generated by proteins, lipids and carbohydrates was observed, representing a total of 0.51 KJ ind^{-1} consumed, from which more than 48 % (0.24 KJ ind^{-1}) corresponded to proteins, 46 % (0.23 KJ ind^{-1}) corresponded to total lipids and about 6 % (0.03 KJ ind^{-1}) being provided by carbohydrates. In the fed larvae, from the beginning of the larval period until the onset of the metamorphosis (day 19) there was a gradual increase in energy generated from proteins, total lipids and carbohydrates. It should be noted that from the onset of metamorphosis there was a 25 % and 20 % loss of total energy per individual, in larvae fed T-iso and C.cal, respectively.

The energy differentials for each component (KJ ind^{-1}) at each different period of larval development are present in Table 5.5. During the development from eggs to early D-veliger larvae stage (day 0 to 2) a negative differential was observed for the energy provided by total lipids, indicating that this component was consumed during this process, while during metamorphosis (day 19 to 23) negative differentials were observed for all components. In the larvae fed with T-iso the most important energy contribution to metamorphosis came from both proteins and total lipids. Carbohydrates also contributed, but to a lesser extent. However, in the larvae fed with C.cal the most important energy contribution to metamorphosis came from total lipids.

5.4. Discussion

Larval forms often develop from small eggs with low energy content and are thus presumed to become dependent on exogenous food sources at an early stage of development. Endogenous egg reserves are known to be important for survival and growth throughout embryogenesis until exogenous food sources become available (Ojea et al., 2008). In the present study, larvae of *R. decussatus* survived for an extended period of time without access to algal food. The high survival rate of unfed larvae until day 5 after fertilization (76.78 %) suggests that egg reserves can contribute to the maintenance of larvae beyond the period of embryonic development. Even after 23 days of starvation (end of record), about 23 % of the larvae were still swimming and did not show any signs of exhaustion. Apparently, larvae of *R. decussatus*, similarly to those of *C. gigas* (Moran and Manahan, 2004), have the capacity to survive without phytoplankton for long periods of time. During periods of low phytoplankton availability, larvae may rely on other external sources of energy for the maintenance of their metabolism until phytoplankton food becomes available and

growth can occur. According to Moran and Manahan (2004) and Tang et al. (2005), unfed larvae of *C. gigas* and *Meretrix meretrix*, respectively, are not fully dependent upon their endogenous reserves to support maintenance metabolism. In our experiment, larvae were maintained in 0.45 µm filtered seawater and could not have met their energy needs by phytoplankton or any large detrital material. Our results support the idea that *R. decussatus* larvae have complemented their nutritional requirements by alternative sources of energy such as dissolved organic material (DOM) in seawater or bacteria as suggested by several authors (Manahan, 1990; Gallager et al., 1994; Gomme, 2001). An uptake of dissolved organic materials from seawater has been documented for many species of marine invertebrates (Gomme, 2001), and has been suggested to be a potentially important source of energy for larvae (Manahan, 1990). Due to larval mortality in the culture tanks, populations of bacteria or heterotrophic protists may have grown in the larval cultures between water changes and might have been used as food by the surviving larvae.

Larvae fed with monospecific diets showed a rather gradual decrease in survivorship with time. This decrease was a little bit more pronounced between days 14 and 19, when metamorphosis with major morphological and physiological changes starts and movements and feeding are reduced or inhibited.

Larval length growth rates of *R. decussatus* from hatching to metamorphosis obtained in the fed treatments (ranged between 4.93 and 5.27 µm day^{-1}) were similar to those reported in previous work by our team (Matias et al. 2009) and also by Beiras and Albentosa (2004) and Ojea et al. (2008), suggesting that both monospecific diets used in the experiment had an adequate nutritional quality. However, significant differences were observed between the two diets in terms of length, length growth rates and organic matter growth rate. The diet of T-iso seems to be more adequate since larvae accumulated significantly more organic matter reserves in their tissues, which has allowed them to overcome more successfully the critical phase of metamorphosis (75.66 % larvae with foot). A decreased in organic matter was observed between day 19 and 23 (metamorphosis). A similar decrease in the organic content of larvae during metamorphosis had been cited by Videla et al. (1998) in *Ostrea chilensis*, by Rodríguez et al. (1990) and Labarta et al. (1999a,b) in *Ostrea edulis* and by Moran and Manahan (2004) in *C. gigas*. The larvae fed with C.cal presented the highest length, suggesting that larvae fed with these microalgae seem to allocate more energy to the biosynthesis of the shell, which might have affected the success of the metamorphosis (42.81 % larvae with foot). However, the percentage of metamorphosed larvae for both diets was higher than those reported by Zine et al. (1998), Ojea et al. (2008) and Matias et al. (2009). During starvation an increase in shell length of the clam larvae was observed. This phenomenon has also been described in other bivalves and is related to an increase in shell mass (Laing and Child, 1996). It seems that the biosynthesis of the shell is a priority in the distribution of energy resources, even under extreme nutritional stress. One of the main effects of starvation in invertebrates is a decrease in metabolism down to maintenance levels (Mayzaud, 1976; Albentosa et al., 1996). Under such conditions, energy is provided by body reserves and/or the catabolism of

tissues. Thus, one of the first consequences of starvation is a loss of organic matter, as this was observed in the present study during the 23 days of food deprivation, especially following day 5 after fertilization. A similar decrease in organic matter has also been observed when the quantity or quality of the food provided is inadequate (Albentosa et al., 2002).

In the present study, proteins and total lipids were found to be the most important biochemical constituents of *R. decussatus* larvae, independent of the feeding regime, while carbohydrates (consisting at about 60 % of free reducing sugars and at about 40 % of polysaccharides) were present in lower quantities. Similar results have been reported for the early developmental stages of other bivalve species (*e.g.* Videla et al., 1998; Labarta et al., 1999b; Tang et al., 2005; Chaparro et al., 2006). In absolute terms, carbohydrates showed a slight increase in both monospecific diets, while proteins and total lipids increased considerably after the early stages, reflecting an increased ingestion of particulate food. It should be noted that the increase for each biochemical component was not continuous, presenting different rates according to the stage of development. Larvae fed with T-iso presented higher total lipids and protein contents than larvae fed with C.cal, however no significant differences were observed between diets in terms of biochemical composition. In unfed larvae, in contrast, a decrease of proteins and total lipids was detected, while carbohydrates remained almost constant. These results suggest that total lipids and proteins are the major energy sources during larval development of *R. decussatus*, unlike the situation in adults where glycogen represents the principal energy reserves (Albentosa et al., 2007). This is also evident in the unfed larvae, in which the catabolism of proteins and total lipids contributed most to the larval metabolism, especially after day 5. Also the high negative energy differentials observed, mainly in proteins and total lipids, in the starved larvae during the whole larval development of *R. decussatus* highlight this evidence. Neutral lipids (energetic lipids) were the most abundant lipid constituents of larvae of *R. decussatus* and the main energy source, since in the monospecific diets neutral lipids were accumulated in greater amounts than phospholipids after the onset of feeding, indicating that the catabolism of endogenous neutral lipids was rapidly offset by food intake during the nutritional transition. Moreover, in conditions of total starvation, energetic lipids decreased more strongly than structural lipids (phospholipids).

In the family Ostreidae, larvae undergoing metamorphosis do not feed, since they have already lost the velum while gills have not yet developed. Thus, larvae in this stage use stored proteins and/or neutral lipids as shown by the decrease in the energy contribution of these components (Ferreiro et al., 1990; Rodríguez et al., 1990; Videla et al., 1998; Labarta et al., 1999a). Some authors have supported the idea that lipids, especially neutral lipids, are the principal energy substrate during larval development and metamorphosis (*e.g.* Holland and Hannat, 1974; Ferreiro et al., 1990). However, Rodríguez et al. (1990) stated that proteins were more important than lipids in metamorphosis and suggested the lack of agreement being due to differences in experimental conditions. The results of our experiment conciliate both hypotheses. While lipids (especially neutral lipids) were found to be the main source of energy during

embryogenesis to the early D-veliger larvae, proteins are probably the most important substrate during metamorphosis.

Whyte et al. (1992) in their work on *Crassadoma gigantea* establish that the larvae best equipped to begin the process of metamorphosis present an energy content of around 6.1-6.3 KJ g^{-1}, while the minimum premetamorphic capacity of the larvae is between 4.5 and 5.0 KJ g^{-1}. Indeed, in our study, the larvae fed with T-iso, with an energy content registered immediately before metamorphosis of 9.95 KJ g^{-1} were able to successfully begin metamorphosis (75.66 %). On the other hand, the larvae fed with C.cal which presented a value of 3.98 KJ g^{-1}, slightly inferior to the minimum premetamorphic suggested by Whyte et al. (1992), presented considerable lower levels of metamorphosis success (42.81 %). Moreover these results support the fact that the T-iso diet is more adequate for *R. decussatus* larvae rearing than the C.cal one.

Our results indicate that eggs reserves could energetically contribute to the maintenance of larvae beyond the embryonic phase. Obviously, the transition from the endotrophic to the exotrophic phase in the early development of *R. decussatus* larvae is a gradual process, with the existence of an intermediate, mixotrophic phase, in which yolk reserves and planktonic particles are used simultaneously by young larvae. The endotrophic phase largely corresponds to the first two days after fertilization. It is followed by a mixotrophic phase from day 2 to approximately days 5-8, after which the larvae are exclusively exotrophic. The mixo- and exotrophic phases involve a period of growth with deposition of the prodissoconch II shell as well as increase in the visceral mass and in velum size. Thereafter, around day 20, metamorphosis occurs, starting with the development of the foot and the primary gill filaments and culminating in larval settlement. This intense morphogenetic activity is supported by energy originating from proteins which are accumulated during the planktonic larval phase.

Beyond its contribution to our general knowledge of the biochemical dynamics in the early developmental phases of *R. decussatus*, the present study provides some useful information for hatchery production programs of this clam: 1. Among monospecific algal diets, T-iso seems to be more adequate than C.cal. 2. Food should not be administered until after day 2 following fertilization (endotrophic phase), and thereafter food supply should be rather moderate until day 8 (mixotrophic phase).

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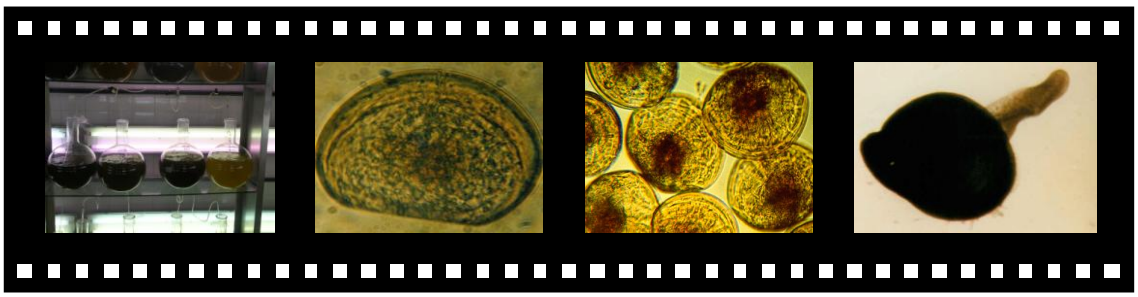
Chapter 6

The influence of different microalgal diets on European clam (*Ruditapes decussatus*, Linnaeus, 1758) larvae culture performances

Matias D., Ben-Hamadou R., Joaquim S., Ramos M., Sobral P., Leitão A. The influence of different microalgal diets on European clam (*Ruditapes decussatus*, Linnaeus, 1758) larvae culture performances. Aquaculture (submitted).



Abstract

The European clam, *Ruditapes decussatus* is a species with high commercial importance in Portugal and other Southern European countries. However, the development of *R. decussatus* culture has been limited by the patterns of natural recruitment. In response to the environmental constraints of wild stock, and growing demand for clams, the development of hatchery technology will provide the aquaculture industry with an alternative reliable source of clam spat. However, and despite an indisputable growing know-how in mollusks hatchery, the larval stages remain critical stages in the life cycle of bivalves, some biological aspects are still unknown and beyond them bivalve feeding requirements are poorly understood. Food quality is extremely important in larval cohort success. A key to this success would be the identification of nutritional regimes that result in maximum growth, survival and settlement which in turn would reduce hatchery operating costs. The present study determined the effect of six different nutritional regimes: unfed (negative control); two monospecific diets – *Isochrysis aff galbana* (100 cells μl^{-1}) and *Chaetoceros calcitrans* (100 cells μl^{-1}) and three bispecific mixture of both microalgae at different proportions – 50/50, 60/40 and 40/60 cells μl^{-1} in equivalent volume, on the survival, growth, settlement and biochemical composition of hatchery reared larvae of *R. decussatus* aiming to provide crucial information on the nutritional requirements and the optimisation of feeding practices. The results clearly demonstrate that the different nutritional regimes originated variations in mortality, growth, metamorphic rate and larval biochemical composition. A holistic approach incorporating all physiological response showed that the bispecific diet based on flagellate *I. aff galbana* and the diatom *C. calcitrans* in a proportion of 60/40 cell μl^{-1} was the more adapted for the development from 2-day-old larvae to postlarvae. Moreover, the monospecific diet *I. aff galbana* provided an overall good performance. The results clearly demonstrate that *R. decussatus* larvae cannot use *C. calcitrans* with the same efficiency as *I. aff galbana* at such early stages of development; however the inclusion of *C. calcitrans* improved late larval development. Specific recommendations to achieve optimal larval growth, based on our results, can be established: during a first period (2 to 5 days after fertilization), raise larvae with *I. aff galbana* monospecific diet, followed by (5 to 17days after fertilization) the bispecific diet *I. aff galbana* plus *C. calcitrans* with the flagellate in the highest proportion and then during late larval period on *I. aff galbana* plus *C. calcitrans* with the diatom in the highest proportion. These results constitute an important first step in the hatchery larval nutrition *R. decussatus* and a prerequisite for future work on the improvement of larval development and the optimisation of feeding practices that will maximise larvae yield and minimise cost in aquaculture hatcheries.

Keywords: Bivalve larvae; *Ruditapes decussatus*; Growth; Biochemical composition; Diet; Larval nutrition.

6.1. Introduction

The clam *Ruditapes decussatus* is distributed from the south and west coast of the British Isles to the Mediterranean and along the Atlantic coast of Morocco and Senegal (Puigcerver, 1996). In Portugal, it is found mainly in the south, in the Ria Formosa Lagoon and represents the most economically important bivalve species, representing 27 % of national annual aquaculture production and 64 % of the shellfish production (DGPA, 2009). However the development of *R. decussatus* culture has been limited by the patterns of natural recruitment that are highly variable from year to year and can be influenced by a number of environmental and biological factors (Matias et al., 2009; Marshall et al., 2010). As a response to environmental and biological constrains of wild stock, and growing demand for clams, the development of hatchery technology will provide the aquaculture industry with an alternative reliable source of clam spat. Despite an indisputable growing know-how in mollusks hatchery, the larval stages are presently thought to be the most critical stages in the life cycle of bivalves, some biological aspects are still unknown and beyond them bivalve feeding requirements are poorly understood (Helm et al., 2004; Rico-Villa et al., 2006). Optimizing hatchery production, namely larval stages is therefore, an important task since hatchery production economics are largely dependent on limiting larvae mortality. The importance of optimizing larvae nutrition is a crucial aspect of overall hatchery operations (Widdows, 1991). Food quality is indeed extremely important in larval cohort success (Powell et al., 2002).

Microalgae are the primary food source used in aquaculture as live feeds for all growth stage of bivalves (Brown et al., 1997). Consequently, bivalve development is closely related to the quantity and quality of phytoplankton available. To be used as food for bivalve larvae, microalgae present specific characteristics such as an adequate size for ingestion (less than 10 µm with an optimal range between 2 to 5 µm), no thick cell wall, a good nutritional value (adequate biochemical composition) and, for practical and economical reasons, must be relatively easily produced (Robert and Trintignac, 1997). Meeting the specific diet requirements of bivalve larvae depends not only on the concentration, but also on composition of food (Baldwin and Newell, 1995). Due to the fact that mixed algal diet increases the probability of achieving an equilibrate diet, microalgae are generally supplied for bivalves in plurispecific rations without however a clear understanding of their need in essential components (Muller-Fuega et al., 2003). Usually, each experimental or commercial hatchery has own microalgae mixtures, which changes throughout larval development (Coutteau and Sorgeloos, 1992). Feeding mixed diet of at least one type of flagellate and one type of diatom has been shown to produce optimal growth and development of bivalve larvae (*e.g.* Galley et al., 2009; Pettersen et al., 2010). Consistently commercially species used in hatcheries worldwide typically include the flagellate *Isocrhysis aff galbana* (Clone T-iso) and the diatom *Chaetoceros calcitrans*; both have good nutritional proprieties as feed for many aquaculture organisms (*e.g.*Gouda et al., 2006). The quality of the food provided to culture bivalve

larvae is indeed a critical factor in the future quality and health of the larvae, and is a major contributor to the success of aquaculture operations (Brown, 2002; Gouda et al., 2006). A key to this success is the identification of regimes that result in maximum growth, survival and settlement which in turn will reduce hatchery operating costs.

The role of each microalgae on larval development and metamorphosis is known for a number of commercially important and produced bivalves (*e.g. Pecten maximus* (Delaunay et al., 1993); *Crassostrea gigas* (Rico-Villa et al., 2006); *Mytillus galloprovincialis* (Pettersen et al., 2010)), however such basic and essential information is still unavailable for *R. decussatus* larvae.

The objective of the present study was then to determine the effect of different diets (monospecific and bispecific in different proportions) on the survival, growth, settlement and biochemical composition of hatchery reared larvae of the European clam *R. decussatus*.

6.2. Materials and Methods

6.2.1. Microalgae

Microalgae *I. aff galbana*, *Skeletonema costatum* and *C. calcitrans* were initially cultured in 250 ml Erlenmeyer flasks and then in 2 l, 10 l glass carboys and finally in 80 l plastic bags, in batch cultures. The microalgae culture from the 80 l volume was devoted to broodstock conditioning while the one from10 l was used for larvae. Seawater (salinity = 36±1) was filtered (0.45 µm), UV-treated, enriched with sterilised f/2 medium (Guillard, 1975), in a temperature-controlled room at 20±2 ºC under continuous illumination. For diatom culture sodium metasilicate was added as silica source. A continuous aeration was provided to enhance growth and prevent the algae from settling. Microalgae were harvested when the culture reached the end of the exponential growth phase. Before being used, algal densities were determined daily by standard algal cell counts (Büker chamber).

6.2.2. Broodstock conditioning, spawning and larval production

Genitors of *R. decussatus* (>35 mm shell length) were collected from Ria de Aveiro (40º42′N; 08º40′W) (western coast of Portugal). Eighty individuals were placed in 50 l tanks for 2 months in flow through system at 20±1 ºC with a daily phytoplankton supply equivalent to 4 % of the dry weight of the clams' soft tissue (Utting and Millican, 1997). Three microalgae species were supplied in equal quantity and equivalent volume: *I. aff galbana*, *S. costatum* and *C. calcitrans*. The water was enriched with this mixed diet and distributed to the tanks at a flow rate of 0.6 to 0.8 l min^{-1}. Clams were induced to spawn by a rapid increase of temperature from 20 ºC to 28±1 ºC over a 6 h interval. Oocytes from all females that spawned (n=6) were pooled and mixed with

sperm (about ten spermatozoids per oocyte) from males that spawned (*n*=8) for fertilization. Fertilized eggs were collected on a sieve, washed with filtered (0.45 µm) and UV-treated seawater. Embryos were put to incubate in 250 l cylinder-conical tanks at a density of 100 embryos ml^{-1}. Seawater was regulated at 22±1 ºC. At the end of 48 h incubation, the D-larvae were collected on a 30 µm mesh screen and the total number of veligers was calculated based on three 1 ml aliquots. The D-larvae were then used in the experiment.

6.2.3. Experimental design

The influence of food quality on larval rearing, during D-larvae until day 21, was estimated by feeding larvae with six nutritional regimes, in triplicate: unfed (negative control) (Unfed); two monospecific diet – *I. aff galbana* (100 cells μl^{-1}) [T(100)] and *C. calcitrans* (100 cells μl^{-1}) [C(100)] and a bispecific mixture of *I. aff galbana* plus *C. calcitrans* at a different proportions – 50+50 [T(50)+C(50)], 60+40 [T(60)+C(40)] and 40+60 [T(40)+C(60)] cells μl^{-1}. The early D-larvae collected were dispensed at an initial density of 10±2 larvae ml^{-1} in 5 l tanks with natural filtered (0.45 µm) and UV-treated seawater with a salinity of 36±1 and temperature at 22±1 ºC. Food was added daily to each tank and water was renewed every 2-3 days. Over the larval rearing period and at each water renewal, larvae were recovered by draining the tanks. They were counted to estimate survival and to detect presence of foot and/or any morphological alterations in the velum. The presence of a foot was scored to determine larval development status; larvae that showed a clearly visible foot bulging out of the shell (pediveligers) and lost its velum were considered metamorphosed. At each water renewal, a sample of larvae was taken in order to estimate mean shell length. Antero-posterior shell length was measured for 50 randomly sampled larvae from each replicate using an ocular micrometer.

Samples of 5,000 larvae each were taken for biochemical analyses on days 2, 5, 13, 17 and 21. Remaining larvae were transferred to the tanks. Also, biochemical composition of the microalgae *I. aff galbana* and *C. calcitrans* was carried out on days 2, 5, 13 and 17 of the experimental period, in triplicate. Both type of samples were centrifuged, resuspended and rinsed with iso-osmotic ammonium formate (3 % w/v) to remove the salt, stored at -20 ºC and freeze-dried prior to biochemical analysis (proteins, total lipids, phospholipids, neutral lipids, carbohydrates, polysaccharide, free reducing sugar and organic matter).

6.2.4. Biochemical composition of microalgae and larvae

A micro-analytical fractionated extraction scheme developed by Holland and Gabbott (1971) and Holland and Hannant (1973) was followed for the determination of the contents of biochemical components. Lyophilized samples were homogenized in 500 µl distilled water using a sonicator. Samples were sonicated in an ice water bath for three intervals of 10 s at 20 W each to obtain

a thoroughly homogenized sample. Separate samples (200 µl) of the initial homogenate were taken for analyses of proteins, carbohydrates, total lipids, neutral lipids and free reducing sugar.

Total lipid content was extracted by the method of Bligh and Dyer (1959) and taken up in 500 µl chloroform and were determined by the methods of Marsh and Weinstein (1966) using tripalmitin as a standard, and the absorbance was determined at 375 nm. Neutral lipids were determined in the same way as total lipids; 200 µl samples of neutral lipids in chloroform were dried for 20 min at 100 ºC and used for determinations. Phospholipids were determined as the difference between total and neutral lipids.

Proteins were precipitated by cold 5 % trichloroacetic acid (TCA) and the precipitate washed in warm 1.0 N NaOH. Protein concentration was assayed by the method of Lowry et al. (1951), modified by Bensadoun and Weinstein (1976) and Hess et al. (1978), at 750 nm using serum albumin as a standard.

Hydrolysed and unhydrolysed samples of TCA supernatant were used for the determination of total carbohydrates and free reducing sugars by a modification of the method of Folin and Malmros (1929). The components were quantified with a ferricyanate reduction reaction at 420 nm using glucose as a standard. Polysaccharides were determined as the difference between carbohydrates and free reducing sugars.

The organic matter was calculated as the sum of proteins, total lipids and carbohydrates.

Energy conversion factors used for lipid, carbohydrate and protein were 35.24, 17.16 and 18.00 KJ g^{-1}, respectively (Beukema and De Bruin, 1979).

6.2.5. Statistical analyses

Percentage of growth increment (shell length and organic matter) between sampling times was calculated using the formula: $GI = [(G_{i+1}-G_i)/ G_i]*100$; where GI is the percentage of growth increment between sampling times; G_i and G_{i+1} the mean shell length or mean organic matter at respectively sampling time *i* and *i*+1. Growth rate (shell length and organic matter) between sampling times was calculated using the formula: $K = (X_{i+1}-X_i)/\Delta t$; whereby K is the growth rate (shell length and organic matter) per sampling time; X_i and X_{i+1} are the mean shell length or mean organic matter at respectively sampling time *i* and *i*+1, Δt is the time interval in days between sampling *i* and *i*+1. Linear regressions were fitted to shell length and organic matter over larval growth trajectories to determine length and organic matter growth equation from early D-larvae to metamorphic larvae for each treatment.

Survival of *R. decussatus* larvae from the different groups was examined using the Kaplan-Meier method. The Mantel-Cox test was used to compare the survival trends between groups. Differences in survival (end of experimental period), growth (shell length, organic

matter, shell length and organic matter growth rate, growth increment in shell length and organic matter and growth rate in shell length and organic matter as a slope of the growth equations) and biochemical composition (proteins, total lipids, phospholipids, neutral lipids, carbohydrates, polysaccharide and free reducing sugar) were tested by analyses of variance (ANOVA) or Kruskal–Wallis ANOVA on ranks, whenever the assumptions of ANOVA failed among days of the same nutritional regime and between regimes. $P<0.05$ was considered to indicate a statistically significant result. Multiple pair comparisons among means were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test. Percentage data were arcsine transformed to normalize variance (Sokal and Rohlf 1981).

To ordinate the diets performances in terms of biochemical features a Principal Component Analysis (PCA) using the increment of biochemical composition ratios were done. Ratios increments were calculated for periods between each sampling day (periods: day 2 to 5, 5 to 13, 13 to 17 and 17 to 21). The proportions between proteins/organic matter (Prot/OM), total lipids/organic matter (TL/OM), neutral lipids/total lipids (NL/TL), total lipids/proteins (TL/Prot), phospholipids/total lipids (PHL/TL), polysaccharides/carbohydrates (PS/CH) and proteins energy/total energy (Prot Ener/TE) were the considered biochemical composition ratios increments. These ratios were selected from all biochemical composition combinations possible using the equivalent vectors method, "EVM" (Hamadou et al., 2001).

All statistical analysis was performed using either Sigmastat 3.5 or MATLAB® 7.1 (www.mathworks.com).

6.3. Results

6.3.1. Biochemical composition of the microalgae

The biochemical composition and organic matter of the different diets are presented in the Table 6.1. The diet T(100) presented the higher values of proteins, total lipids, neutral lipids and organic matter, followed by the diet T(60)+C(40). The diet C(100) showed the higher values of carbohydrates and free reducing sugars, followed by the diet T(40)+C(60).

Table 6.1. Biochemical composition profile (mean ± SD, *n*=12) of the different nutritional regimes (unfed; *Isochrysis aff galbana* (100 cells μl^{-1}) [T(100)]; *Chaetoceros calcitrans* (100 cells μl^{-1}) [C(100)] and a bispecific mixture of both microalgae at a different proportions – 50/50 [T(50)+C(50)], 60/40 [T(60)+C(40)] and 40/60 [T(40)+C(60)] cells μl^{-1}.

Biochemical composition (ng 100 cel^{-1})	Nutritional regimes					
	Unfed	T(100)	C(100)	T(50)+C(50)	T(60)+C(40)	T(40)+C(60)
Proteins	_	0.81±0.06	0.23±0.04	0.52±0.03	0.57±0.04	0.46±0.03
Total Lipids	_	0.40±0.08	0.10±0.03	0.25±0.04	0.28±0.05	0.22±0.03
Carbohydrates	_	0.15±0.03	0.22±0.03	0.18±0.02	0.18±0.02	0.19±0.02
Neutral lipids	_	0.18±0.02	0.07±0.02	0.12±0.02	0.14±0.02	0.11±0.02
Phospholipids	_	0.22±0.08	0.04±0.01	0.09±0.01	0.15±0.05	0.11±0.01
Free reducing sugars	_	0.05±0.01	0.14±0.02	0.09±0.02	0.08±0.00	0.10±0.01
Polysaccharides	_	0.10±0.03	0.09±0.02	0.09±0.02	0.09±0.02	0.09±0.02
Organic matter	_	1.36±0.13	0.55±0.08	0.96±0.05	1.04±0.07	0.88±0.04

6.3.2. Survival and growth

A low survival rate, not unusual in this *R. decussatus* culture life phase, was observed over the study period for all nutritional regime [10±2 % in unfed to 48±10 % in T(60)+C(40)]. Survival rate of larvae developed under different nutritional regimes, using Kaplan-Meier method, is presented in Figure 6.1. No significant differences in the survival rate trends were observed among treatments (Mantel-Cox test; $P>0.05$). However, the comparison between nutritional regimes at the end of the experimental period showed that larvae fed with T(60)+C(40) presented significantly higher survival rates than unfed larvae and larvae fed with C(100) (K–W., $H=22.01$, $df=5$, $P\leq0.007$).

R. decussatus larvae reared under the different nutritional regimes exhibited varying growth (shell length and organic matter) over the 21 days of the experiment (Table 6.2). The growth rate and growth increments of unfed larvae showed slight increase in shell length and a sudden decrease in organic matter, especially following day 5 after fertilization. When expressed as change in organic matter, negative values for growth increments and rates were obtained, indicating a progressive decrease in the organic tissue of unfed larvae. In contrast, larvae reared with the monospecific and bispecific diets showed an increase in shell length and also in organic matter, with the highest values of shell length (211.9±29.3 µm) and organic matter (444.6±18.8 ng $larvae^{-1}$) observed in the larvae fed with T(60)+C(40) at the end of the experimental period. Shell length presented significant differences among all nutritional

regimes (K–W., *H*=1905.51, *df*=5, *P*≤0.001), with the exception of larvae fed with T(50)+C(50) *versus* larvae fed with T(40)+C(60). Concerning organic matter significant differences were only found between fed and unfed larvae (K–W., *H*=37.35, *df*=5, *P*≤0.001), with the exception of C(100).

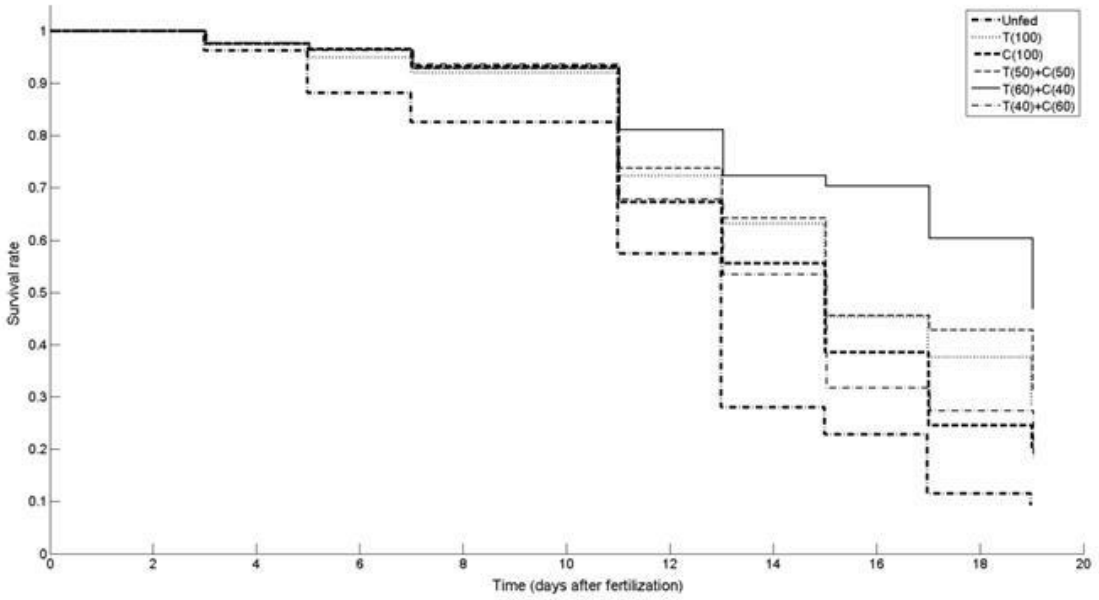


Figure 6.1. Survival rate of *Ruditapes decussatus* larvae fed with different nutritional regime (unfed; two monospecific diet – *Isochrysis aff galbana* (100 cells µl^{-1}) [T(100)] and *Chaetoceros calcitrans* (100 cells µl^{-1}) [C(100)] and a bispecific mixture of both microalgae at a different proportions – 50/50 [T(50)+C(50)], 60/40 [T(60)+C(40)] and 40/60 [T(40)+C(60)] cells µl^{-1} using the Kaplan-Meier method.

The shell length growth rate showed highly random variations between sampling days in all nutritional regimes. In general, this rate trend to decrease during the experimental period. The larvae fed with T(100) had the highest shell length growth rate at days 5 (7.50±0.35 µm day^{-1}) and presented significant higher shell length growth rate than unfed larvae (K–W., *H*=14.32, *df*=5, *P*=0.014). The larvae fed with T(60)+C(40) showed the highest value at day 13 (7.05±0.60 µm day^{-1}), 17 (4.88±2.45 µm day^{-1}) and 21 (4.65±2.35 µm day^{-1}). Significant differences were observed between fed and unfed larvae at day 13 and the larvae fed with T(60)+C(40) presented significant higher shell length growth rate than the other nutritional regimes (ANOVA, *F*=105.23, *df*=5, *P*≤0.001), no significant differences were observed between nutritional regimes at days 17 and 21 (ANOVA, *P*>0.05). In all fed larvae the organic matter growth rate showed an erratic variation, decreasing and increasing throughout the experimental period. The larvae fed with T(60)+C(40) presented the highest organic matter growth rate at all sampling times (day 5 - 41.39±9.97 ng larvae^{-1} day^{-1}; day 13 - 11.40±4.51 ng larvae^{-1} day^{-1}; day 17 - 29.38±11.85 ng larvae^{-1} day^{-1} and day 21 - 20.65±10.48 ng larvae^{-1} day^{-1}). Significant differences were only observed at day 5 between T(100) and unfed (K–W., *H*=15.69, *df*=5,

P=0.008), at day 13 between T(60)+(C40) and unfed (ANOVA, *F*=4.81, *df*=5, *P*=0.014), at day 17 between fed and unfed larvae (ANOVA, *F*=9.67, *df*=5, *P*≤0.001). No significant differences were observed between nutritional regimes at day 21 (K-W, *P*>0.05).

Concerning the shell length growth increment, the larvae fed with T(100) showed the highest increment at day 5 (23.27±1.09 %) and the larvae fed with T(60)+(C40) presented the highest level at days 13 (48.22±8.68 %) and 17 (11.23±6.47 %), while larvae fed with T(40)+C(60) showed the highest length growth increment at day 21 (10.33±1.56 %). Significant differences were found between unfed larvae and larvae fed with T(100) at day 5 (K–W., *H*=15.56, *df*=5, *P*=0.008). At day 13, the larvae fed with T(60)+C(40) presented significant higher increment than the other nutritional regimes and significant differences were also observed between fed and unfed larvae (ANOVA, *F*=61.79, *df*=5, *P*≤0.001). No significant differences were observed between nutritional regimes at days 17 and 21 (ANOVA, *P*>0.05). The highest value of organic matter growth increments was observed in larvae fed with T(60)+C(40) at days 5 (426.92±102.86 %) and 21 (24.20±15.74 %), T(50)+C(50) at day 13 (65.77±50.69 %) and C(100) at day 17 (85.65±7.97 %). However, significant differences were found between unfed, T(100) and T(60)+C(40) at day 5 (K–W., *H*=15.71, *df*=5, *P*=0.008) and between fed and unfed larvae at day 17 (ANOVA, *F*=18.15, *df*=5, *P*<0.001). No significant differences were observed between nutritional regimes at days 13 and 21 (ANOVA, *P*>0.05).

The linear growth (shell length and organic matter) and respective growth equations for all nutritional regimes are presented in Figure 6.2. In the fed larvae, the growth equation that showed the highest slopes for both shell length (5.52 µm) and organic matter (19.19 ng larvae^{-1}) was the one of the larvae fed with T(60)+C(40), indicating that larvae fed with this regime presented the higher growth. On the contrary, the larvae fed with C(100) showed the lowest slopes in both the equation of shell length (3.55 µm) and organic matter (7.59 ng larvae^{-1}). In terms of shell length only significant differences were observed between the unfed larvae and larvae fed with T(60)+C(40) (K–W., *H*=13.21, *df*=5, *P*=0.021). In terms of linear organic matter significant differences were observed, with the exceptions of larvae fed with the regimes T(40)+C(60), T(50)+C(50) and T(100) (ANOVA, *F*=104.68, *df*=5, *P*<0.001).

The metamorphic rate attained higher values in larvae fed with T(60)+C(40) at day 19 (35.0±4.9 %) and 21 (73.5±3.3 %) (Table 6.3). At day 19 significant differences were observed between T(60)+C(40) and all other nutritional regimes. Also, at day 19 the metamorphic rate was significantly higher in T(100) comparatively with unfed, C(100), T(50)+C(50) and T(40)+C(60) (ANOVA, *F*=43.38, *df*=5, *P*<0.001). In day 21 no significant differences were observed between T(60)+C(40) *versus* T(100) and C(100) *versus* T(40)+C(60) (ANOVA, *F*=382.05, *df*=5, *P*<0.001).

Table 6.2. Shell length (µm) (mean±SD, *n*=150), shell length growth rate (µm day^{-1}) (mean±SD, *n*= 3), relative length growth increment (%)(mean±SD, *n*= 3), organic matter (ng larvae^{-1}) (mean±SD, *n*=9), organic matter growth rate (ng larvae^{-1} day^{-1}) (mean±SD, *n*=3) and relative organic matter growth increment (%) (mean±SD, *n*= 3) of *Ruditapes decussatus* larvae fed with different nutritional regimes (unfed; *Isochrysis aff galbana* (100 cells µl^{-1}) [T(100)]; *Chaetoceros calcitrans* (100 cells µl^{-1}) [C(100)] and a bispecific mixture of both microalgae at a different proportions – 50/50 [T(50)+C(50)], 60/40 [T(60)+C(40)] and 40/60 [T(40)+C(60)] cells µl^{-1}.

Food regime	Days after fertilization	Shell length (µm)	Shell length growth rate (µm day^{-1})	Shell length growth increment (%)	Organic matter (ng larvae^{-1})	Organic matter growth rate (ng larvae^{-1} day^{-1})	Organic matter growth increment (%)
Unfed	2	96.7±5.9	-	-	29.1±0.0	-	-
	5	100.2±5.7	1.50±0.46	3.59±2.09	37.9±3.9	2.93±1.29	30.17±13.28
	13	101.4±5.6	0.42±0.02	3.42±0.24	35.1±4.0	-0.34±0.98	-5.94±19.32
	17	102.3±6.1	0.16±0.00	0.64±0.00	22.5±4.6	-3.14±2.15	-34.13±22.03
	21	104.5±6.7	0.44±0.94	4.32±0.00	19.1±1.5	-0.85±1.42	-12.47±20.58
T(100)	2	96.7±5.9	-	-	29.1±0.0	-	-
	5	119.2±7.0	7.50±0.35	**23.27±1.09**	149.1±14.7	40.01±4.91	412.74±50.67
	13	162.8±8.8	5.45±0.18	36.59±1.25	194.6±6.2	5.68±1.91	31.35±13.91
	17	174.1±12.8	3.08±1.50	7.60±3.67	306.7±33.8	28.03±7.65	57.53±14.82
	21	188.9±19.6	2.57±1.24	5.97±3.09	317.0±14.0	2.57±6.84	3.99±9.61
C(100)	2	96.7±5.9	-	-	29.1±0.0	-	-
	5	104.3±6.5	2.52±0.27	7.82±0.84	75.1±12.2	15.35±4.07	158.32±42.02
	13	144.7±10.0	5.05±0.12	38.75±1.16	96.3±11.4	2.65±2.70	48.10±31.24
	17	161.2±14.8	4.04±0.28	11.14±0.72	178.2±14.1	20.47±0.69	**85.65±7.97**
	21	169.6±24.5	1.04±1.06	5.68±1.70	191.4±7.8	3.31±3.65	7.86±9.61
T(50)+C(50)	2	96.7±5.9	-	-	29.1±0.0	-	-
	5	112.5±4.7	5.26±0.31	16.31±0.97	95.8±10.1	22.24±3.38	229.44±34.83
	13	152.1±10.3	4.96±0.29	35.28±2.34	155.6±30.9	7.46±5.05	**65.77±50.69**
	17	165.0±10.7	3.20±1.18	8.44±3.19	236.6±35.0	20.25±5.67	53.56±19.19
	21	181.9±12.3	3.95±0.92	9.62±2.40	265.1±9.2	7.14±6.95	13.50±14.49
T(60)+C(40)	2	96.7±5.9	-	-	29.1±0.0	-	-
	5	117.3±10.9	6.88±1.35	21.34±2.02	153.2±29.9	41.39±9.97	**426.92±102.86**
	13	173.8±16.2	7.05±0.60	**48.22±8.68**	244.5±6.6	11.40±4.51	64.32±37.08
	17	193.3±28.2	4.88±2.45	**11.23±6.47**	362.0±49.3	29.38±11.85	48.00±19.36
	21	211.9±29.3	4.65±2.35	9.54±4.42	444.6±18.8	20.65±10.48	**24.20±15.74**
T(40)+C(60)	2	96.7±5.9	-	-	29.1±0.0	-	-
	5	111.6±4.4	4.95±0.67	15.36±2.09	87.7±0.7	19.52±0.23	201.39±2.32
	10	147.2±9.0	5.35±0.31	38.39±2.85	120.0±4.1	4.00±0.53	36.56±5.06
	13	154.4±10.5	2.38±0.31	6.16±0.81	219.5±18.9	24.95±5.39	83.73±19.75
	21	180.8±20.4	4.24±0.68	**10.33±1.56**	262.1±18.4	10.66±4.16	19.71±8.12

The values in bold type are the most relative length and organic matter growth increment observed at each sampling time.

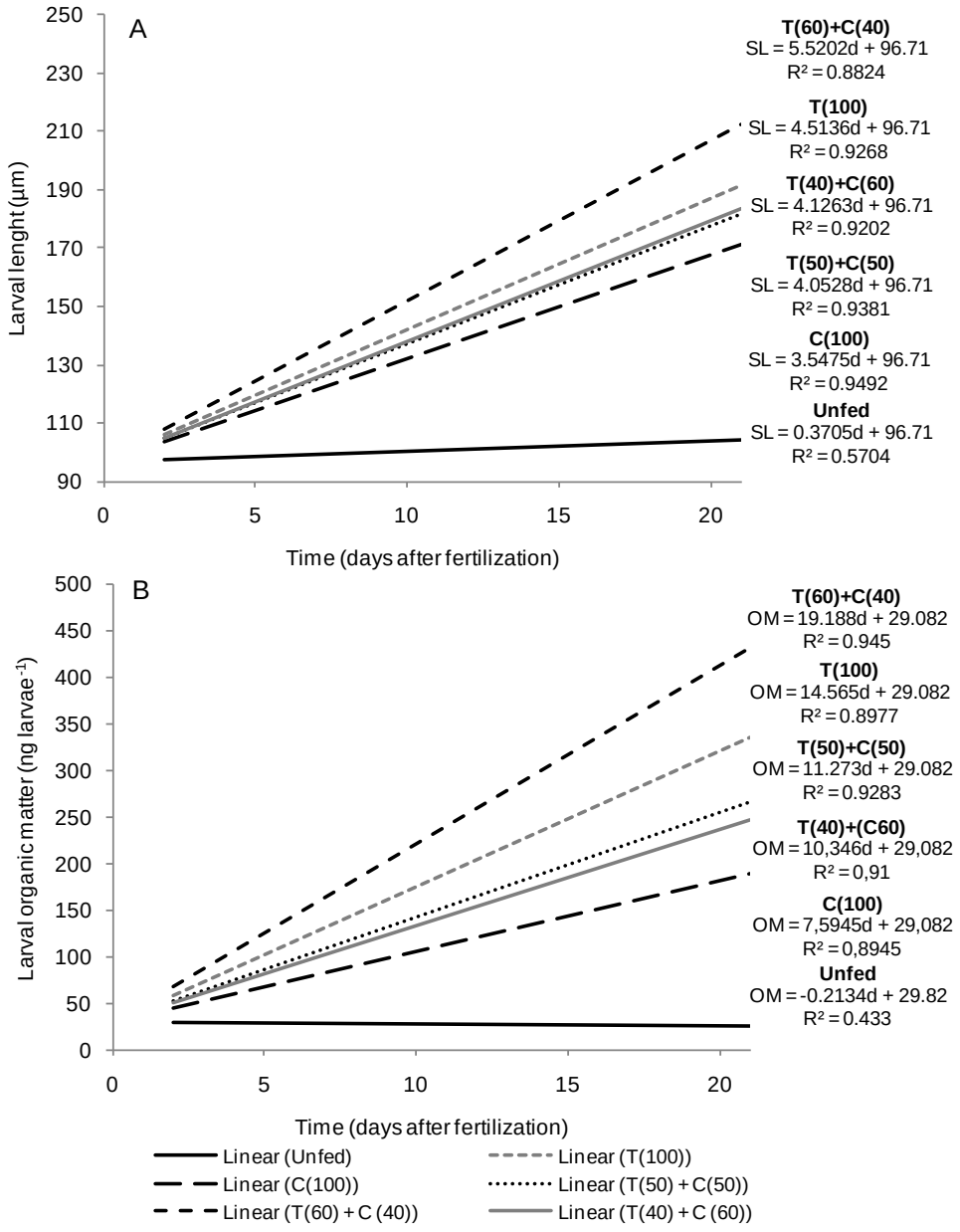


Figure 6.2. The linear growth (A - shell length and B- organic matter) and respective growth equations of *Ruditapes decussatus* larvae fed with different nutritional regime (unfed; two monospecific diet – *Isochrysis aff galbana* (100 cells μl^{-1}) [T(100)] and *Chaetoceros calcitrans* (100 cells μl^{-1}) [C(100)] and a bispecific mixture of both microalgae at a different proportions – 50/50 [T(50)+C(50)], 60/40 [T(60)+C(40)] and 40/60 [T(40)+C(60)] cells μl^{-1}.

Table 6.3. Percentage of metamorphic rate (mean±SD, *n*=9) of *Ruditapes decussatus* larvae development under different nutritional regimes (unfed; *Isochrysis aff galbana* (100 cells μl^{-1}) [T(100)]; *Chaetoceros calcitrans* (100 cells μl^{-1}) [C(100)] and a bispecific mixture of both microalgae at a different proportions – 50/50 [T(50)+C(50)], 60/40 [T(60)+C(40)] and 40/60 [T(40)+C(60)] cells μl^{-1}.

Food regime	Days after fertilization	Metamorphic rate (%)
Unfed	19	0.0±0.0
	21	0.0±0.0
T(100)	19	18.4±7.5
	21	65.5±2.5
C(100)	19	1.7±1.5
	21	37.3±4.8
T(50)+C(50)	19	6.7±1.2
	21	48.4±3.7
T(60)+C(40)	19	35.0±4.9
	21	73.5±3.3
T(40)+C(60)	19	2.2±0.9
	21	32.0±1.9

6.3.3. Biochemical composition of the larvae

Table 6.4 shows the evolution of proteins, total lipids and carbohydrates throughout the larval development period expressed as ng $larvae^{-1}$. In the unfed culture, the protein content showed a slight initial increase between days 2 and 13, followed by an abrupt decrease to a minimum value of 11.14±5.36 ng ind^{-1} on day 21. A similar situation was observed for total lipids; the level increase till day 5 followed by a gradual decline till the end of the experiment (day 2: 7.34±0.00 ng ind^{-1}; day 23: 1.72±1.27 ng ind^{-1}), while carbohydrates remained almost constant throughout the experimental period. In the unfed larvae the total losses observed in the gross biochemical composition represent a decrease of 34.36 % in total organic matter. Proteins and total lipids presented a tendency to increase in fed larvae overall the experimental period. Larvae fed with T(60)+C(40) presented the higher increase in all three biochemical components followed by larvae fed with T(100). Significant differences were observed in proteins between unfed and fed larvae. Larvae fed with T(60)+C(40) presented significantly higher protein contents than larvae fed with C(100), T(50)+C(50) and T(40)+C(60) (K–W., H=71.39, df=5, $P \leq 0.001$). Total lipids only showed significant differences between fed and unfed larvae (K–W., H=33.21, df=5, $P \leq 0.001$). Carbohydrates showed a different behaviour in larvae fed with monospecific and bispecific diets: in larvae fed with T(100) and C(100) irregular variations in carbohydrate contents were detected; in larvae fed T(50)+C(50), T(60)+C(40) and

T(40)+C(60) carbohydrates increase till day 17, but decreased between days 17 and 21. Once more, significant differences were detected between fed and unfed larvae. The larvae fed with T(60)+C(40) showed significant higher carbohydrates than larvae fed with C(100), T(50)+C(50) and T(40)+C(60) and also the larvae fed with the monospecific diet T(100) showed significant higher carbohydrate levels than larvae fed with the monospecific C(100) (K-W., H=89.08, df=5, P≤0.001). In term of percentage of energy, proteins and total lipids were the main contributors to the total energy. The energy generated by proteins and total lipids was approximately 44 % and 43 % of the total energy respectively whereas carbohydrates contributed only with around 13 %.

The results for neutral lipids, phospholipids, free reducing sugars and polysaccharides in larvae under the six nutritional are shown in Table 6.5. In general, the main contribution to total lipids came from neutral lipids. During the development of the larvae keep without food (Unfed), a considerable reduction in energetic lipids (neutral lipids) was observed (from 5.89±0.29 to 0.76±0.34 ng ind^{-1}) while structural lipids (phospholipids) increased until day 5 (from 1.45±0.00 to 4.27±1.72 ng ind^{-1}) and subsequently decrease till the end of experiment. The larvae fed with T(60)+C(40) showed the greatest increase throughout the experimental period in both neutral lipids and phospholipids, followed by the larvae fed with T(100). Significant differences were observed in neutral lipids (K–W., H=68.33, df=5, P≤0.001) and phospholipids (K–W., H=20.75, df=5, P≤0.001) between unfed and fed larvae, excepting between unfed and the diets C(100) and T(40)+C(60) in phospholipids contents. Free reducing sugars levels in fed larvae generally increased until day 17 and subsequently decreased till the end of experiment. Larvae fed with T(60)+C(40) presented the highest increase in free reducing sugars followed by larvae fed T(100). The polysaccharide contents of fed larvae showed different behaviour among diets; no general trend could be established. In the unfed culture, free reducing sugars and polysaccharide contents remained almost constant overall the experimental period. Significant differences were observed in free reducing sugars (K–W., H=31.26, df=5, P≤0.001) and polysaccharides (K–W., H=37.35, df=5, P≤0.001) between unfed and fed larvae. In terms of free reducing sugars, larvae fed with the diets T(100) and T(60)+C(40) presented significant higher values than larvae fed with C(100). Also, significant differences were observed between larvae fed with T(60)+C(40) and the larvae fed with T(50)+C(50).

Table 6.4. Principal biochemical composition (mean±SD, *n*= 9) expressed in ng larvae^{-1} and percentage energy equivalents of the principal biochemical component (mean±SD, *n*=9) during *Ruditapes decussatus* larval development with different nutritional regimes (unfed; *Isochrysis aff galbana* (100 cells µl^{-1}) [T(100)]; *Chaetoceros calcitrans* (100 cells µl^{-1}) [C(100)] and a bispecific mixture of both microalgae at a different proportions – 50 /50 [T(50)+C(50)], 60/40 [T(60)+C(40)] and 40/60 [T(40)+C(60)] cells µl^{-1}.

Food regime	Days after fertilization	Gross biochemical composition					
		Proteins		Total lipids		Carbohydrates	
		ng larvae^{-1}	% Total energy	ng larvae^{-1}	% Total energy	ng larvae^{-1}	% Total energy
Unfed	2	13.86±0.14	43.08±0.00	7.34±0.00	44.66±0.00	4.14±0.14	12.26±0.00
	5	20.22±3.92	46.47±2.96	9.72±0.83	43.93±2.43	4.36±0.41	9.59±0.53
	13	21.90±5.66	59.04±2.26	5.58±0.83	29.56±1.14	4.36±0.40	11.40±1.77
	17	12.15±4.67	52.77±13.94	3.38±1.26	30.13±13.10	4.03±0.40	17.10±1.06
	21	11.14±5.36	62.26±13.00	1.72±1.26	18.87±13.00	3.51±0.20	18.87±1.65
T(100)	2	13.86±0.14	43.08±0.00	7.34±0.00	44.66±0.00	4.14±0.14	12.26±0.00
	5	70.61±13.95	46.37±15.99	21.58±3.13	28.16±5.70	40.32±4.18	25.47±1.75
	13	103.63±17.00	49.80±4.59	35.91±10.34	33.52±8.07	36.21±6.65	16.68±3.77
	17	131.55±11.49	72.33±3.07	108.99±17.61	3.56±0.92	46.26±9.47	24.11±2.82
	21	136.93±9.26	33.46±1.48	120.30±2.53	57.59±0.14	38.29±4.57	8.95±1.33
C(100)	2	13.86±0.14	43.08±0.00	7.34±0.00	44.66±0.00	4.14±0.14	12.26±0.00
	5	29.97±8.53	31.14±5.85	17.16±2.19	38.86±8.17	25.18±7.81	27.00±5.54
	13	50.15±8.53	45.01±9.93	21.30±8.95	35.79±10.05	22.96±4.86	19.19±1.14
	17	83.45±6.56	38.28±2.15	53.56±4.38	47.98±1.02	31.77±8.92	13.74±2.84
	21	93.21±4.42	38.65±3.54	66.52±6.21	53.81±3.16	19.24±5.65	7.54±1.99
T(50)+C(50)	2	13.86±0.14	43.08±0.00	7.34±0.00	44.66±0.00	4.14±0.14	12.26±0.00
	5	36.37±2.97	34.28±3.51	22.40±1.72	41.15±1.14	27.59±5.11	24.57±3.49
	13	71.34±11.80	41.49±10.60	39.50±20.88	41.90±13.02	30.53±6.56	16.61±2.47
	17	96.23±8.73	35.58±2.84	72.87±10.54	52.48±3.12	33.99±4.41	11.94±0.39
	21	109.35±5.26	35.16±0.65	92.44±5.97	58.17±1.18	21.72±2.03	6.67±0.61
T(60)+C(40)	2	13.86±0.14	43.08±0.00	7.34±0.00	44.66±0.00	4.14±0.14	12.26±0.00
	5	70.00±15.57	43.75±13.54	24.33±1.72	30.38±4.47	43.97±12.61	25.87±3.95
	13	125.49±8.07	47.65±2.86	45.84±3.44	34.03±2.22	50.96±15.97	18.32±3.96
	17	186.03±28.54	46.70±6.37	83.90±30.98	39.98±7.96	54.68±3.31	13.32±3.14
	21	216.30±12.95	41.17±3.34	138.22±15.79	51.34±3.89	41.23±5.38	7.49±0.81
T(40)+C(60)	2	13.86±0.14	43.08±0.00	7.34±0.00	44.66±0.00	4.14±0.14	12.26±0.00
	5	23.58±9.18	24.51±10.06	21.02±2.08	42.74±4.02	33.07±6.46	32.75±6.50
	13	47.13±7.91	35.69±7.23	27.09±6.25	39.73±7.02	34.05±6.52	24.58±3.93
	17	98.59±12.81	55.97±2.46	56.87±10.03	33.44±1.37	36.27±11.59	10.59±3.68
	21	138.61±18.88	46.57±3.55	72.04±4.16	47.46±3.16	18.59±1.03	5.97±0.50

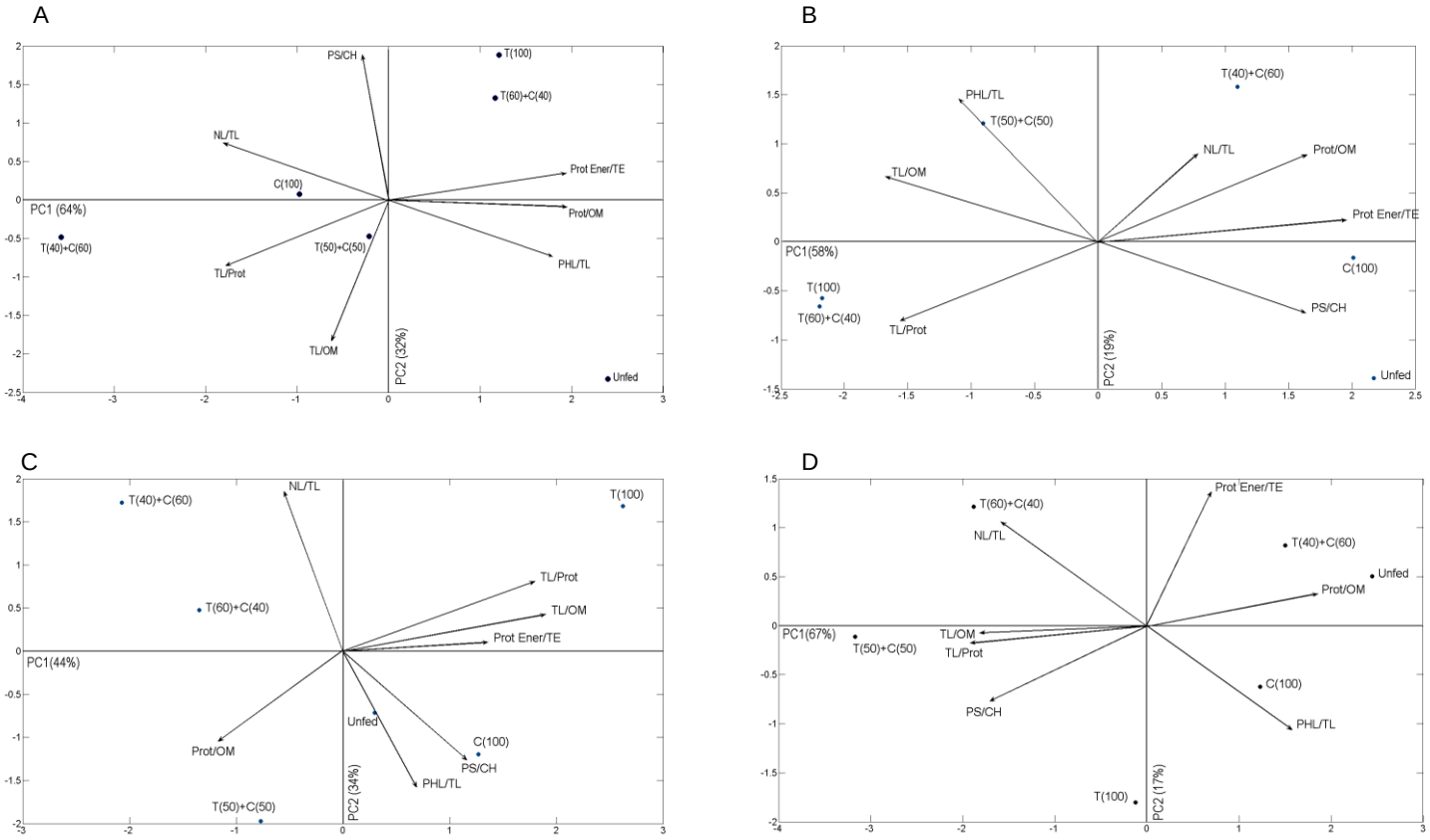
Table 6.5. Neutral lipids, phospholipids, free reducing sugars and polysaccharides (mean±SD, *n*=9) expressed in ng larvae^{-1} during *Ruditapes decussatus* larval development with different nutritional regimes (unfed; *Isochrysis aff galbana* (100 cells µl^{-1}) [T(100)]; *Chaetoceros calcitrans* (100 cells µl^{-1}) [C(100)] and a bispecific mixture of both microalgae at a different proportions – 50/50 [T(50)+C(50)], 60/40 [T(60)+C(40)] and 40/60 [T(40)+C(60)] cells µl^{-1}.

Food regime	Days after fertilization	Biochemical composition (ng larvae^{-1})			
		Neutral lipids	Phospholipids	Free reducing sugars	Polysaccharides
Unfed	2	5.89±0.29	1.45±0.00	3.35±0.13	0.78±0.00
	5	5.44±1.22	4.27±1.72	3.71±0.15	0.65±0.30
	13	3.65±1.00	1.93±0.24	3.31±0.15	1.04±0.11
	17	1.44±0.74	1.93±1.19	3.05±0.16	0.98±0.39
	21	0.76±0.34	0.97±1.04	2.99±0.20	0.52±0.18
T(100)	2	5.89±0.29	1.45±0.00	3.35±0.13	0.78±0.00
	5	16.20±1.84	5.38±4.66	25.57±1.78	14.75±2.15
	13	21.56±2.44	14.40±10.07	22.96±2.28	13.25±5.37
	17	77.00±7.86	31.99±18.82	30.33±4.42	15.92±9.12
	21	81.97±11.45	38.33±14.86	23.41±8.30	19.87±11.21
C(100)	2	5.89±0.29	1.45±0.00	3.35±0.13	0.78±0.00
	5	11.37±2.96	5.79±4.70	20.35±1.39	9.40±4.43
	13	18.54±1.00	6.62±7.60	12.97±2.66	9.99±5.46
	17	33.71±8.35	19.85±5.38	14.21±2.67	17.56±8.76
	21	31.23±5.07	35.30±8.40	9.84±0.69	9.39±5.78
T(50)+C(50)	2	5.89±0.29	1.45±0.00	3.35±0.13	0.78±0.00
	5	14.13±4.81	8.27±2.90	18.59±1.03	9.00±5.83
	13	29.57±5.13	21.92±12.87	22.31±1.86	8.22±6.11
	17	58.80±11.73	14.06±3.95	28.90±6.07	5.09±3.11
	21	58.66±5.42	33.78±2.63	10.49±1.15	11.23±0.41
T(60)+C(40)	2	5.89±0.29	1.45±0.00	3.35±0.13	0.78±0.00
	5	15.51±1.38	8.82±0.86	33.00±0.88	18.11±3.46
	13	30.66±3.57	15.17±1.26	43.19±7.20	13.41±3.46
	17	36.74±5.75	47.15±27.69	41.68±5.70	12.99±6.39
	21	96.86±5.01	41.36±17.61	27.17±11.02	12.82±9.83
T(40)+C(60)	2	5.89±0.29	1.45±0.00	3.35±0.13	0.78±0.00
	5	15.51±5.10	5.52±4.31	26.74±3.65	9.69±0.69
	13	23.09±10.48	7.24±3.22	28.18±3.47	8.61±3.60
	17	38.40±10.33	28.54±8.77	22.37±2.69	19.87±0.97
	21	42.12±3.41	29.92±2.28	14.28±1.273	4.31±1.47

At each sampling period (days 2 to 5, 5 to 13, 13 to 17 and 17 to 21), a Bi-plot Principal Component Analysis was applied to ordinate in the same reduced space, the six nutritional regime and the biochemical composition ratios increments of the larvae: Prot/OM, TL/OM, NL/TL, TL/Prot, PHL/TL, PS/CH and Prot Ener/TE (Figure 6.3). The obtained plans were composed by the first 2 principal components and were considered sufficient to learn from the data about interrelationships between biochemical composition ratios increments and nutritional regimes. These bi-plots represent the dual projection of objects scores (nutritional regimes) and variable vectors. Vectors pointing in the same direction corresponded to increments of biochemical composition ratios which are highly correlated. Increments of biochemical composition ratios explaining or inducing the discrepancy between nutritional diets were those well correlated to PC1 and PC2 (*i.e.* presenting high scores as vector projection to the corresponding PC). Between days 2 to 5 the PC1 describing 64 % of the original information was highly positive correlated to Prot/OM and Prot Ener/TE and also correlated to PHL/TL. By opposition, PC1 was negatively correlated to NL/TL and TL/Prot suggesting that PC1 ordinate positively the nutritional regimes which induce a gain in proteins and phospholipids and a loss in neutral lipids. PC2 describes 32 % of data information and was highly positively correlated to PS/CH and by opposition negatively correlated to TL/OM. PC2 separates the nutritional regimes with high increments in polysaccharides and low increments in total lipids. The nutritional regimes were clearly separated in four groups T(100) with T(60)+C(40) showing positive scores in both PC1 and PC2; C(100) with T(50)+C(50) displaying slightly negative scores for PC1 and intermediate scores for PC2; T(40)+C(60) with considerable negative score for PC1 and unfed located in the negative side of both PC1 and PC2. Between days 5 to 13 the PC1 describing 58 % of the original information was highly positive correlated to Prot Ener/TE and also correlated to Port/OM and PS/CH. PC1 was negatively correlated to TL/Prot and TL/OM suggesting that PC1 ordinate positively the nutritional regimes which induce a gain in proteins and polysaccharides and a loss in total lipids. PC2 describes 19 % of data information and was positively correlated to PHL/TL and NL/TL, suggesting that PC2 ordinate the nutritional regimes with high increments in phospholipids and neutral lipids. The nutritional regimes were clearly separated in four groups T(100) with T(60)+C(40) displaying highly negative scores for PC1 and slightly negative scores for PC2; C(100) with unfed showing highly positive scores for PC1 and negative scores for PC2; T(50)+C(50) displaying positive score for PC1 and slightly negative score for PC2; T(40)+C(60) with positive scores in both PC1 and PC2. Between days 13 to 17 the PC1 describing 64 % of the original information was highly positive correlated to TL/OM and TL/Prot and also with Prot Ener/TE. PC1 ordinate the nutritional regimes which induce a gain in total lipids. PC2 describes 34 % of data information and was highly positively correlated to NL/TL and by opposition negatively correlated to PHL/TL, PS/CH and Prot/TL. PC2 separates the nutritional regimes with high increments in neutral lipids and low increments in phospholipids and polysaccharides. The nutritional regimes were clearly separated in four groups T(100)

showing highly positive scores in both PC1 and PC2; C(100) with unfed displaying average positive scores in both PC1 and PC2; T(50)+C(50) with slightly negative score for PC1 and considerable negative score for PC2; T(40)+C(60) with T(60)+C(40) located in the negative side for PC1 and positive side for PC2. At the end of the experiment (between days 17 to 21) the PC1 describing 67 % of the original information was highly positive correlated to Prot/OM. PC1 was negatively correlated to TL/Prot and TL/OM suggesting that PC1 ordinate positively the nutritional regimes which induce a gain in proteins and a loss in total lipids. PC2 describes 17 % of data information and was positively correlated to Prot Ener/TE and NL/TL and by opposition negatively correlated to PHL/TL and PS/CH. PC2 separates the nutritional regimes with high increments in neutral lipids and proteins energy and low increments in polysaccharides and phospholipids. The nutritional regimes were clearly separated in five groups T(40)+C(60) with unfed showing positive scores in both PC1 and PC2; C(100) with average score in PC1 and slightly negative score in PC2; T(100) displaying neutral score in PC1 and highly positive score in PC2; T(50)+C(50) showing highly negative score for PC1 and slightly negative score for PC2; T(60)+C(40) displaying moderate negative score for PC1 and highly positive score for PC2.

Figure 6.3. Biplot Principal Component Analysis of the six nutritional regime (unfed; monospecific diets – *Isochrysis aff galbana* (100 cells µl-1) [T(100)] and *Chaetoceros calcitrans* (100 cells µl-1) [C(100)] and a bispecific mixture of both microalgae at a different proportions – 50/50 [T(50)+C(50)], 60/40 [T(60)+C(40)] and 40/60 [T(40)+C(60)] cells μl^{-1}) and the biochemical composition ratios increments [proteins/organic matter (Prot/OM), total lipids/organic matter (TL/OM), neutral lipids/total lipids (NL/TL), total lipids/proteins (TL/Prot), phospholipids/total lipids (PHL/TL), polysaccharides/carbohydrates (PS/CH) and proteins energy/total energy (Prot Ener/TE)] of the *Ruditapes decussatus* larvae at each sampling period (A - days 2 to 5; B - 05 to 13; C - 13 to 17 and D - 17 to 21).



6.4. Discussion

Bivalve production in hatchery is undeniably related to the quality of food provided (Helm et al., 2004). With the present hatchery techniques, microalgae production can represent 30 % to 40 % of the spat cost in hatchery (Rico-Villa et al., 2006). Achieving optimal algal composition for larvae bivalve feed which will allow optimum performances has been the object of extensive nutritional studies for many aquaculture bivalve species (*e.g.* Tang et al., 2005; Gouda et al., 2006; Rico-Villa et al., 2006; Liu et al., 2009; Pettersen et al., 2010), however such information was unavailable, until know, for *R. decussatus* larvae. Effectively, the effect of microalgal diet on larval performance is difficult to generalize and seems to be species-specific and sensitive to algal culture conditions (Pernet and Tremblay, 2004). Experiment developed in this study was carried out to improve hatchery efficiency of *R. decussatus* by assessing the nutritional value of two different common microalgae used in bivalve hatcheries, *I. aff galbana* and *C. calcitrans* used as monospecific and bispecific diet in different proportions.

The biochemical composition of cultured microalgae was comparable to those previously reported by Fernandéz-Reiriz et al. (1989), with the exception of the proportion of carbohydrates in *C. calcitrans*, however biochemical composition of microalgae has been shown to vary considerably, with the culture conditions (*e.g.* Thompson et al., 1996; Pattersen et al., 2010).

Survival is an important parameter in evaluating culture conditions and consequently the availability of bivalve larvae for further aquaculture operations (Baldwin and Newell, 1995). The choice of microalgae used as food strongly affects the survival of planktothrophic bivalve larvae (Gallager et al., 1986; Baldwin and Newell, 1995; Gouda et al., 2006). In the present study, larvae fed with six different nutritional regimes showed a gradual decrease in survivorship with a similar trend over all experimental time. In the fed larvae, this decrease was more pronounced after day 10, when the endogenous egg reserves were completely consumed. Also the high survival rate observed on the unfed larvae until day 5 after fertilization confirm the results previously observed by our team that egg reserves can contribute to the maintenance of larvae beyond the period of embryonic development (Matias et al., 2011). In general, at the end of the experimental period, the flagellate diets (predominantly or in equal proportion) [T(60)+C(40); T(100); T(50)+C(50)] originated better larval survival than the predominantly diatom diets. Effectively, the diet T(60)+C(40) showed a significant higher level of survival than C(100) and the unfed nutritional regime. Thus, the feeding diets composed essentially by the flagellate *I. aff galbana* produced the best results for *R. decussatus* larvae in terms of survival rates.

Larvae reared under the six nutritional regimes all presented an increase in shell length growth over time. The shell length growth observed in unfed larvae suggests that the biosynthesis of the shell is a priority in the distribution of energy resources. In extreme

nutritional stress, energy seems to be provided by body reserves and/or the catabolism of tissues with a loss of organic matter, as reflected by the values of organic matter rate and growth increments in the present study. Across all microalgae diets, larvae shell length measured 169 to 211 µm and organic matter attained 191 to 444 ng larvae^{-1} at the end of the experimental period. The flagellate diets (predominantly or in equal proportion) produced significant higher shell length growth and higher organic matter growth than the predominantly diatom diets. The results of linear growth also corroborate these observations. In this study the larval growth results clearly indicate that the diets constituted by *I. aff galbana* are highly nutritional balanced for *R. decussatus.* Indeed, even the larvae fed the flagellate monospecific diet T(100) showed best larval performance than bispecific diets with the diatom in equal or higher proportions.

Tang et al. (2005) and Gouda et al. (2006) suggested that the effect of a diet on the growth of larvae during the early larval period is small, since larvae use stored energetic contents and dissolved organic matter uptake, algal diets not playing a key role in growth during this period. However, in general in this study we found the most significant variations in growth rate and growth increment especially in early days (until day 17) suggesting that besides the use of eggs' energetic reserves and dissolved organic matter, the diet has a preponderate effect on *R. decussatus* early larvae stages performance. Growth rate of larvae fed with the monospecific diet *C. calcitrans* was very low untill day 13, this could be due to the fact that larvae until this date are unable to feed on this species, not because of its cellular size, but due to its peculiar morphology with silica rods that could avoid ingestion during the first days of culture. In terms of the shell length growth increment at each sampling days results showed that T(100) was the better diet between days 2 to 5, the diet T(60)+C(40) was significantly the best diet between day 5 to 13 and also between days 13 to 17 and between the days 17 to 21 the more adequate diet was T(40)+C(60). On the other hand, the organic matter growth increment showed that T(60)+C(40) was the better diet between days 2 to 5, T(50)+C(50) was more adequate between days 5 to 13, C(100) presented the higher levels between days 13-17 and the diet T(60)+C(40) was again the best diet between the days 17 to 21. In general it is possible to infer that during early larval development, larvae preferably select *I. aff galbana* than *C. calcitrans*. According to these evidences and aiming to obtain favourable larval performances different food regimes should be provided during larval development. Our results suggest that the most adequate diet between days 2 to 5 would be the monospecific diet *I. aff galbana* (100 cell µl^{-1}), then between days 5 to 17 it will be necessary to introduce the diatom *C. calcitrans*, so the best diet will be *I. aff galbana* (60 cell µl^{-1}) plus *C. calcitrans* (40 cell µl^{-1}). After this time the results of growth increment suggest that the flagellate should be reduce, so the best diet will be will be *I. aff galbana* (40 cell µl^{-1}) plus *C. calcitrans* (60 cell µl^{-1}). However, studies being evaluate this proposal associated to the evaluation of algal cells ingestion and preference will be developed.

The ability of *R. decussatus* larvae to undergo successful settlement is a result of many interrelated factors, with microalgae playing a major role, both as a chemical sign for burring into a substrate (García-Lavandeira et al., 2005) as well as a dietary requirement necessary for complete pediveliger development (Philips, 2002). The diets formulated with the microalgae *I. aff galbana*, mainly T(60)+C(40) and T(100), in this study, revealed to be more adequate since larvae accumulated significantly more organic matter reserves in their tissues, which has allowed them to significantly overcome faster and more successfully the critical phase of metamorphosis (day 19: T(60)+C(40) – 35.0 %, T(100) – 18.4 %; day 21: T(60)+C(40) – 73.5 %, T(100) – 65.5 %). In general, a decreased in organic matter growth rate was observed between day 17 and 21 (period of metamorphosis). A similar decrease in the organic content of larvae during metamorphosis had been observed in previous work by our team for *R. decussatus* (Matias et al., 2011) and by Videla et al. (1998) in *Ostrea chilensis*, by Labarta et al. (1999) in *Ostrea edulis* and by Moran and Manahan (2004) in *Crassostrea gigas*. During metamorphosis the velum degenerates while the gills are not yet functional and filtration capacity is reduced (Baker and Mann, 1994), consequently larvae use energetic reserves to overcome this intense morphogenetic activity leading to a decrease in the organic content.

In the present study, proteins, total lipids and correspondent energy were found to be the preponderant biochemical constituents of *R. decussatus* larvae, while carbohydrates (consisting in around 60 % of free reducing sugars and 40 % of polysaccharides) were present in lower quantities, independently of the biochemical composition of the diets. Similar results have been reported for other larvae bivalve species (*e.g.* Labarta et al., 1999; Tang et al., 2005; Chaparro et al., 2006). In fed larvae as a reflection an increased ingestion of particulate food, proteins and total lipids increased considerably during larval development, while carbohydrates, in general, showed a slight increase until day 5 and then remain almost constant. These observations show that *R. decussatus* larvae do not accumulate carbohydrates as energy source in the same way as proteins and total lipids. Carbohydrates seem to be catabolise immediately for metabolic maintenance, even when the diet comprised a higher proportion of this component. Concomitantly, in unfed larvae, a decrease in proteins and total lipids was observed, while carbohydrates remained almost constant. These results support the hypothesis that total lipids and proteins are the major energy sources during larval development of *R. decussatus,* suggested previously by our team (Matias et al., 2011). It should be noted that the increase of each biochemical component was not continuous, presenting different rates according to the stage of development and attaining different levels according to the feeding regime. Larvae fed with T(60)+C(40) presented higher levels of protein, total lipid and carbohydrate contents than larvae fed with any other nutritional regime. Neutral lipids (energetic lipids) were the most abundant lipid constituents of larvae of *R. decussatus* and the main energy source, since in the fed larvae neutral lipids were accumulated in greater amounts than phospholipids (structural lipids). Moreover, in conditions of total starvation, energetic lipids decreased more strongly than structural lipids

(phospholipids). In a previous study by our team (Matias et al, 2011) it was put in evidence that neutral lipids were the main source of energy during embryogenesis until early D-veliger larvae and that proteins were the most important substrate during metamorphosis. The fact that the diets formulated with the flagellate *I. aff galbana* [mainly T(60)+C(40) and T(100)] presented the higher levels of protein and neutral lipids and that larvae fed with this diets showed the significant higher metamorphic rate reinforces this idea. In this way, the high food value of *I. aff galbana* during larval stage would suggest that *R. decussatus* larvae have high requirement for lipids and proteins, but not for carbohydrates. Additionally, the poor food value of *C. calcitrans* when used as monospecific diet, indicate that this algal species lacks some essential nutrients or present some constrains in terms of digestibility to *R. decussatus* especially in early larval stages.

All the preceding observations concerning larvae biochemical composition were reinforced when we compared, during the four growing experiment periods, the increments of biochemical composition ratios of larvae in the six nutritional regimes. Indeed, it was evident that between days 2 to 5 and 5 to 13 the larvae fed with the diets T(60)+C(40) and T(100) presented similar evolution. Between days 2 to 5, the biochemical ratios increments associated to these larvae are Prot/OM, Prot Ener/TE and PHL/TL reflecting a high protein synthesis and phospholipids (structural lipids) accumulation, characteristic of an intensive growth. The unfed larvae were also associated to these biochemical indexes, however in a opposition of the larvae fed with T(60)+C(40) and T(100) suggesting the importance of the eggs reserves in absence of food. Also, it was evident that the main source of energy came from neutral lipids, specially reflected by the lower ratios of unfed larvae (days 2 to 5 and 5 to 13). Between days 5 to 13, larvae fed with T(60)+C(40) and T(100) were associated to TL/Prot and TL/OM biochemical composition ratios increments, showing an accumulation in total lipids as energetic reserve to use during the metamorphic phase. After this period the effect of the diets was more notorious. The larvae fed with T(40)+C(60) accumulated more neutral lipids possibly due to the lack of proteins in the diet comparatively to the other bispecific diet, consequently, during metamorphosis (days 17 to 21) the larvae also used lipids as main energy source. *R. decussatus* larvae as well catabolised proteins as energy source during the metamorphosis, as evidenced by the loss of this component and the increments of lipids ratios in the larvae fed with the diets T(50)+C(50) and T(60)+C(40) in the days 17 to 21.The larvae fed with C(100) were associated to Prot/OM and Prot Ener/TE ratios only after day 5, period after which the larvae have the capacity to assimilate this monospecific diet. This delay in the assimilation of the diatom is also evident until the end of the experimental period being reflected by a poor performance of larvae fed with this diet in terms of growth and metamorphic rate.

6.5. Conclusion

The results of this study clearly demonstrate that changes in microalgae species proportion in feeding regimes can produce important variations in mortality, growth, metamorphic rate and larval biochemical composition. A holistic approach incorporating all variables showed that the bispecific diet based on the flagellate *I. aff galbana* and the diatom *C. clacitrans* in a proportion of 60/40 cell μl^{-1} was suitable for *R. decussatus* larval demand and allowed an optimum development from 2-day-old larvae to postlarvae. It is clear, from the results of this study, that *R. decussatus* larvae cannot use *C. calcitrans* with the same efficiency as *I. aff galbana* at such early stages of development; however the inclusion of *C. calcitrans* improved late larval development. Moreoveor, the monospecific diet of *I. aff galbana* allowed a good larval performance, mainly during the early larval development. Taking into consideration that the use of a monospecific diet during hatchery production could greatly facilitate routine management, specific recommendations, based on our results, to achieve optimal larval growth, would be to use *I. aff galbana* monospecific diet during a first period (2 to 5 days after fertilization), followed by the bispecific diet *I. aff galbana* plus *C. calcitrans* with the flagellate in a higher proportion (5 to 17days after fertilization) and then during late larval period a diet with *I. aff galbana* plus *C. calcitrans* with the diatom in the higher proportion.

The results of this study provide important information on the nutritional requirements of hatchery reared *R. decussatus* larvae and the optimisation of feeding practices that will maximise larvae yield and minimise cost in aquaculture hatcheries.

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Chapter 7

The impact of environment in the culture performance of the European clam *Ruditapes decussatus* (Linnaeus, 1758)

Matias D., Joaquim S., Falcão, M., Ramos M., Sobral P., Leitão A. The impact of environment in the culture performance of the European clam *Ruditapes decussatus* (Linnaeus, 1758). Estuarine, Coastal and Shelf Science (submitted).

Abstract

The European clam, *Ruditapes decussatus*, is a species of high significant social and commercial interest in European aquaculture, mainly in southern Europe. In Portugal, this species is extensively produced and harvested in the Ria Formosa Lagoon, where clam farming represents an important social and economical sector. However, in the last two decades clam production decreased, due to severe clam mortalities, consequence of environmental condition degradation. The Ria Formosa Lagoon is an ecosystem in which the anthropogenic pressure of the neighbouring areas is highly reflected in its water quality. The environmental quality assessment of clams' ground plots requires the evaluation of integrated biological effects. The clams' physiological and biological aspects are regulated by several natural environmental factors (*e.g.* temperature, oxygen and food availability), but the exposure to pollutants can also interfere. The aim of this study was to determine over a one year period the biological and physiological status of *R. decussatus* from two ground plots presenting highly different environmental conditions. Specimens were collected monthly from March 2008 to March 2009, from a polluted ground plot (GP_2) and a comparatively reference site (GP_1), and abiotic parameters (temperature, salinity, pH and particulate organic matter of water and temperature, organic matter, chlorophyll *a* and phaepigments of sediment), growth, allometry, mortality, condition index, proteins, total lipids, glycogen and total energy of clams were evaluated. Results showed that *R. decussatus* is a suitable model species for environmental studies and the knowledge of organisms' mortality and growth, and changes in biochemical composition, especially in energetic reserves cycles (glycogen and total lipids), as a reflection of reproduction allow us to better understand the impact of the environment on the organisms. Several consistent differences were observed, between the two ground plots, in the biology and physiology of the *R. decussatus* specimens. The most important difference was the reduction of the reproductive capacity in GP_2 that could influence the viability of the culture by causing recruitment failures. The results obtained in this study, support the potential use of this kind of study to design geographic information systems identifying the better and worst productive clam areas, contributing, this way, to provide adequate management strategies.

Keywords: Bivalve; *Ruditapes decussatus*; Reproductive effort; Ria Formosa Lagoon; Environmental effect; Bivalve production.

7.1. Introduction

Marine ecosystems hold ecological and economic importance since they support vital habitats for organisms and sustain several activities. Highly productive areas such as estuaries and coastal lagoons are, however, at risk due to increasing stress from anthropogenic activities such as urbanization, industrialization, intensive agriculture and mass tourism. Complex mixtures of contaminants are continuously released in these systems deteriorating the water quality and imposing severe restrictions to organisms and possibly causing a decrease in natural resources (Monserrat et al., 2007; Cravo et al., 2009; Cravo et al., 2012; Tlili et al., 2012). An example is the Ria Formosa Lagoon, a major coastal lagoon in the Portuguese south coast. The Ria Formosa Lagoon system is traditionally used for leisure and economic activities such as tourism and bivalve exploitation (Vila-Concejo et al., 2002; Cunha et al., 2005; Guimarães et al., 2012). In the Ria Formosa Lagoon, several hotspots of pollution have been identified. The contaminants already known to be present in the water, sediments and biota from specific sites of the lagoon include metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organotin compounds (Cravo et al., 2012 and literature cited therein).

Analysis of the complete array of the contaminants present in the environment is virtually impossible and does not necessarily reflect the deleterious effects upon the biota. In this context, there is an increasing interest in the determination of the influence of the different contaminants on the biota biology and physiology, namely organisms' mortality and growth, and changes in biochemical composition, especially in energetic reserves cycles (glycogen and total lipids), as a reflection of reproduction capacity. The alterations in energetic reserves cycles could reflect the influence of endocrine disruptor compounds (EDCs) on biota physiology, effectively a lot of natural and/or anthropogenic compounds can exert endocrine disruptions (Lintelmann et al., 2003). These factors can be an effective early warning signal to assess the health of aquatic organisms and at last the ecosystem (Cajaraville et al., 2000).

The European clam (*Ruditapes decussatus*) is widely distributed along the coastal and estuarine areas of Europe and Northern Africa and is an important income resource due to its high commercial value (Matias et al., 2011). *R. decussatus* is extensively produced and harvested in the Ria Formosa Lagoon, where clam farming represents an important economical sector. This species is central to aquaculture revenue, in 2007, the national annual production reported reached 2 metric tons (representing 27 % of the total seafood cultured in Portugal), of which approximately 90 % were originated from the Ria Formosa Lagoon (DGPA, 2009). The culture of *R. decussatus* in the Ria Formosa Lagoon is central to the local socioeconomic framework involving, directly or indirectly, more than 4 500 people. There were around 1 600 licensed clam ground plots within the intertidal area of lagoon, covering 470 ha (DGPA, Institutional communication). However, in last two decades clam production decreased, due to severe clam

mortalities, as a result of environmental condition degradation (degradation of sediments, low water renovation and anthropogenic effects), overstocking culture and pathologies.

The environmental quality assessment of clams' ground plots requires the evaluation of integrated biological effects, particularly in environments where complex mixtures of contaminants are present. The physiological and biological aspects of clams are regulated by several natural environmental factors (*e.g.* temperature, oxygen and food availability) (Sobral and Widdows, 1997a; Sobral and Widdows, 1997b; Sobral and Widdows, 2000; Sobral and Fernandes, 2004), being also influenced by the exposure to chemical pollutants (Kraeuter and Castagna, 1989; Sobral and Widdows, 1997c).

The biochemical composition of bivalves has been mainly studied as an indicator of their health status (*e.g.* Albentosa et al., 1996; Fernández-Reiriz et al., 1999) and it is largely dependent on the food supply, state of gonadal development and metabolic activity of the organisms (Beiras et al., 1994; Pérez-Camacho et al., 2003). Energy reserves such glycogen and lipids are of considerable importance in reproduction and seasonal energy storage and utilization in marine bivalve molluscs are closely correlated to environmental conditions and the annual gametogenic cycles (*e.g.* Holland, 1978; Delgado et al., 2004; Tlili et al., 2012). This cycle translates into a seasonal pattern of biochemical composition that can vary according to species and geographical origin (Albentosa et al., 2007; Matias et al., 2009). The exposure of biochemical components to chemical pollutants may also impacts energy balance of bivalves as a result of increased maintenance cost (activation of defence, repair mechanisms and consequently more susceptibility to pathogens) which might lead to further reduction in energy available for metabolic activities like growth and reproduction (Tlili et al., 2012).

Bivalves tolerate a wide range of environmental conditions by adjusting physiological responses in order to achieve maximum rates of growth (Labarta et al., 1997). Fast growth in bivalves is achieved usually by a combination of increased rates of feeding, reduced metabolic rates and lower metabolic costs of growth. However, using energy as a common ecological currency, chemically mediated effects at a cellular level will reduce the energy availability for growth and maintenance for homeostasis and consequently to development processes. Thus, responses related to energy allocation have to be considered, both affecting survivorship of individuals and sustainability of populations (Smolders et al., 2004; Voets et al., 2006).

Biological indices such as the condition index are generally used as tools in the evaluation of nutritional states (Crosby and Gale, 1990) and have applications in the fields of organism recruitment, aquaculture and marine ecology. Condition indexes are of great interest because of the complementary information they can also provide regarding the health status of organisms (*e.g.* Mouneyrac et al., 2008; Cravo et al., 2012; Tlili et al., 2012). Also, allometric relationships, such as length-weight, provide information on the physiological deviation in biological condition of the bivalve (Tlili et al., 2012).

The aim of this study was to compare biological (growth, allometry, mortality and condition index) and physiological (biochemical composition) status of *R. decussatus* individuals collected from two clams' ground plots of the Ria Formosa Lagoon, subjected to different environmental conditions (one in the proximity of pollution sources and with low water renewal and other more clean and with high water renewal). This study, through the understanding of the impact of environmental parameters on the metabolic processes of *R. decussatus*, will contribute to the establishment of adequate ecological and production management strategies.

7.2. Materials and Methods

7.2.1. Studied sites

Ria Formosa Lagoon is a 55 Km long mesotidal lagoon located on the southern coast of Portugal (Algarve) (37º01′N; 07º49′W) with a surface area of approximately 16 300 ha (Constantino et al., 2009). It is a barrier island system comprising mainland, barrier islands, barrier platforms, inlet deltas and shoreface (Gamito, 2006). The Ria Formosa Lagoon does not receive any significant freshwater input, except from a small river (Gilão), presenting several point and diffuse pollution sources from domestic and industrial discharges, agriculture drainages and navigation traffic (Cravo et al., 2012). The present study was undertaken in the mid region of the lagoon, where the most important clam ground plots' area is located. Two clam ground plots contrasting in their environmental characteristics were selected for this study. The ground plot area 1 (GP_1) was chosen as a likely "clean" because it was set up in one of the main channels, closer to the major sea inlet. In contrast, the ground plot area 2 (GP_2) is a relatively protected area with reduced hydrodynamic conditions, located in front of the fishing harbour and recreational pier and known to be affected by contaminants due to intensive navigation traffic and from domestic and industrial discharges (Figure 7.1).

7.2.2. Field sampling

Each experimental ground plot with an area of 50 m^2 was subdivided into 50 quadrats of 1 m^2 to allow a homogeneous distribution of *R. decussatus* spat. A calibrated spat population (mean length: 22.1±3.5 mm and mean live weight: 2.5±1.2 g) obtained from natural banks of Ria Formosa Lagoon was sown at a density of 300 individuals m^{-2}.

Sampling was performed from March 2008 to March 2009 on a monthly basis. Concerning the clams, in each ground plot areas, twenty-five quadrats (0.0625 m^2) were randomly chosen and sampled. The number of live clams was counted to estimate mortality and 75 individuals were

randomly select (three clams from each quadrate) to determine biological parameters (growth, allometry and condition index) and physiological parameters (proteins, total lipids and glycogen).

The physical-chemical characterization of the water was carried out *in situ* by measuring temperature, salinity and oxygen saturation with a multi-parameter probe (YSI ®556 MPS). Sediment temperature was measured using a field thermometer. 6 random replicates of top-layer sediment (0-1 cm) from each plot were collected for determination of sediment organic content, chlorophyll *a* and phaeopigments. A sediment-trap with 6 PVC tubes (5 cm diameter, 50 cm length) was placed on each area for the determination of particulate organic matter and collected monthly. All samples were maintained in the dark and transported to the laboratory in an icebox (≈ 4 ºC).

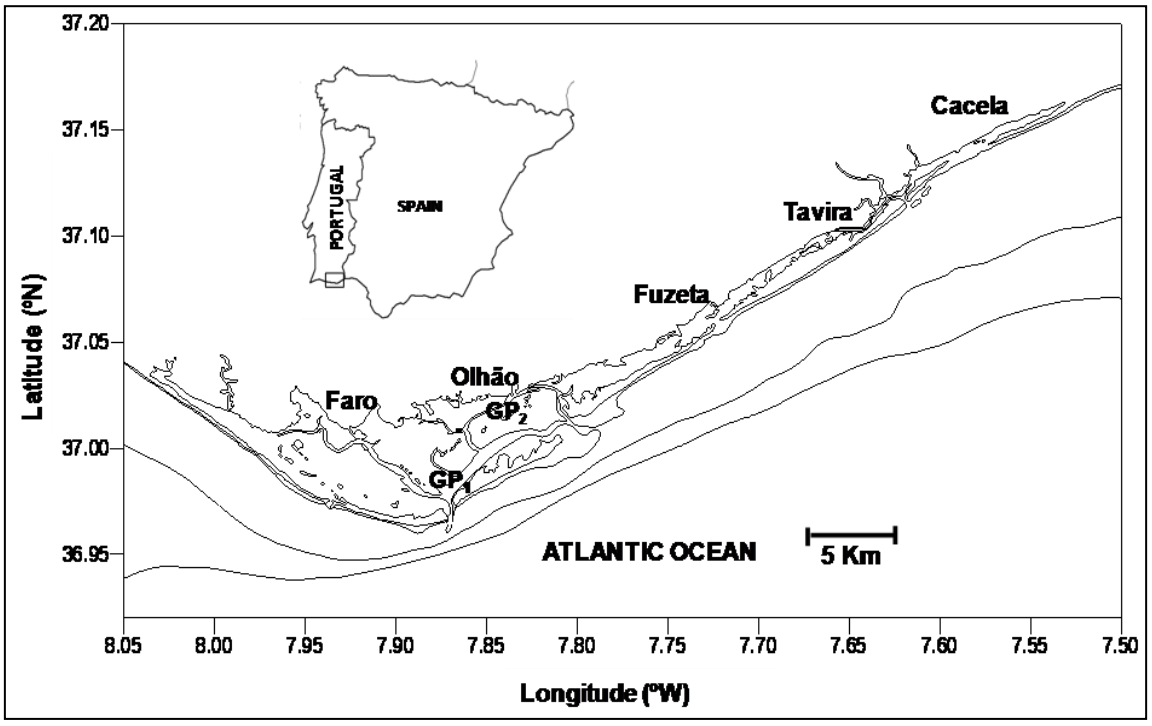


Figure 7.1. Sampling sites within the Ria Formosa Lagoon. Markings represent shellfish ground plots.

7.2.3. Environmental analysis

Sediment organic matter (SOM) and particulate organic matter (POM) was determined by ‘‘loss on ignition’’ at 450 ºC in a muffle furnace, during 4 h (Falcão et al., 2009).

Chlorophyll *a* and pheopigments in the sediment were extracted with acetone (90 %) from the upper sediment layers and determined by spectrophotometry according to Lorenzen (1967) and Parsons et al. (1984).

7.2.4. Growth, allometry and condition index

Clams from both sites (GP_1; GP_2) were individually measured for shell length (maximum antero-posterior distance) accurately to 0.01 mm and total weight was recorded, accurately to 0.01 g. Instantaneous growth rate of length and weight were calculated according to the following expression: $K= \ln (Lt_1/Lt_0)/t$, where L was either length or weight and t was time in days.

The condition index of 25 individual clams was calculated according to Walne and Mann (1975): [Ash free dry weight of meat (g)/dry shell weight (g)]*100. For each sample, the adductor muscles were cut and the clams placed on their ventral surface, allowing them to drain for 5 min. The soft tissues were separated from the shell and both were put in an oven at 80 ºC and weighed after 24 h, then flesh was placed in a muffle furnace at 450 ºC for 24 h and re-weighed to quantify ash.

7.2.5. Clams biochemical quantification

The soft tissue of ten clams of each monthly sample was frozen and stored at −20 °C for biochemical analyses. Each determination of biochemical compounds (proteins, glycogen and total lipids) was carried out in triplicate on pooled material of ten clams. For each pool, protein was determined using the modified Lowry method (Shakir et al., 1994), glycogen content was determined from dried (80 °C for 24 h) homogenate using the anthrone reagent (Viles and Silverman, 1949) and total lipids were extracted from fresh homogenized material in chloroform/methanol (Folch et al., 1957) and estimated spectrophometrically after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966). Values were expressed as a percentage of dry weight (DW). The caloric content of protein, lipid and carbohydrates in tissues was calculated using the factors 17.9 kJ g^{-1} (Beukema and De Bruin, 1979), 33 kJ g^{-1} (Beninger and Lucas, 1984) and 17.2 kJ g^{-1} (Paine, 1971) respectively.

7.2.6. Statistical analysis

Significant differences in environmental parameters during the study period (water and sediment temperature, salinity, pH, dissolved oxygen, particulate organic matter and organic matter, chlorophyll *a* and phaeopigments of the sediment), survival, growth (length and weight), condition index and biochemical composition (proteins, total lipids, glycogen and total energy) were tested using one-way ANOVA. Whenever the assumptions of analysis of variance were not met, the Kruskal–Wallis ANOVA on ranks test was performed. Prior to any analyses, percentage data were arcsine transformed to normalize variance (Sokal and Rohlf, 1981). Multiple pairwise comparisons were performed using the post-hoc parametric Tukey test or the

non-parametric Dunn's test in order to detect significant differences between monthly consecutive samples. In order to assess the influence of bivalves' ground plots on relationships between length and weight, analyses of covariance (ANCOVA) were carried out. Results were considered significant at $P<0.05$. The statistical analyses were undertaken using the SIGMASTAT 3.11 statistical package.

7.3. Results

7.3.1. Environmental parameters

Water and sediment characteristics measured at each site from March 2008 to March 2009 are presented in Table 7.1.

Water and sediment temperature clearly varied during the experimental period, following the typical seasonal variation pattern. The highest values of water temperature were recorded in September 08, at both sites (GP_1 – 24.7 ºC; GP_2 – 25.0 ºC) and the highest sediment temperatures levels at both sites were in August 08 (GP_1 – 25.0 ºC; GP_2 – 24.0 ºC). The lowest temperature values, for both water and sediment, were observed in January 09 (water - 12.4 ºC; sediment – 12.0 ºC) and in December 08 for water – 12.0 ºC and February 09 for sediment – 10 ºC, at GP_1 and GP_2, respectively. No significant differences were observed between ground plots (ANOVA, $P>0.05$).

Salinity ranged from 35.8 (October 08) to 37.0 (February 09) at GP_1 and from 33 (September 08) to 37.1 (February 09) at GP_2. There were no significant differences between ground plots (K-W., $P>0.05$).

The pH varied slightly in both sites, ranging from 7.6 (September 08, GP_2) to 8.6 (August 08, GP_1). The mean pH was highest at GP_1, however no significant differences exist between ground plots (ANOVA, $P>0.05$).

The dissolved oxygen varied considerably between ground plots and sampling months and no particular trend was recorded, however GP_1 presented significant higher values (ANOVA, $F=4.39$, $df=1$, $P=0.047$). Highest dissolved oxygen levels were observed in June 08 (15.5 mg l^{-1}) at GP_2 and in July 08 (15.8 mg l^{-1}) at GP_1. Lowest values were observed in August 08 (5.2 mg l^{-1}) and in January 09 (8.9 mg l^{-1}), at GP_2 and GP_1, respectively.

Particulate organic matter varied noticeably and generally inversely in both sites during the sampling period. Particulate organic matter ranged from 4.1 % (February 09) to 16.3 % (January 09) at GP_1 and from 8.5 % (September 08) to 25 % (October 08) at GP_2. The GP_2 presented significantly higher levels (K-W., $H=7.0$, $df=1$, $P=0.008$).

The highest percentage of sediment organic matter was observed in May 08 (3.1 %) at GP_1 and in June 08 and January 09 (2.4 %) at GP_2. Lowest values were recorded in October 08 and December 08 (0.7 %), at GP_1 and GP_2, respectively. No significant differences were observed between ground plots (K-W., *P*>0.05).

Table 7.1. Environmental parameters measured monthly in water and sediment from the two ground plots (Gp_1 and GP_2) in the Ria Formosa Lagoon between March 2008 and March 2009. WT – water temperature, ST – sediment temperature, S – salinity, DO – Dissolved oxygen, POM – particulate organic matter, SOM – sediment organic matter, Chl a – Chlorophyll *a*, Phaeo – Phaeopigments.

Ground plots	Month	WT (ºC)	ST (ºC)	S	pH	DO (mg l^{-1})	POM (%)	SOM (%)	Chl a (µg g^{-1})	Phaeo (µg g^{-1})
GP1	Mar	16.3	16.0	36.4	8.2	12.6	5.1	1.2	0.2	0.0
	Apr	19.2	18.0	36.0	8.2	13.0	6.2	1.3	3.6	4.2
	May	16.8	17.0	36.1	8.3	12.6	11.5	3.1	6.9	10.8
	Jun	18.2	21.0	36.3	8.5	10.9	6.2	1.4	7.4	5.1
	Jul	23.6	23.0	36.2	8.2	15.8	4.0	1.3	6.5	6.9
	Aug	23.2	25.0	36.4	8.6	10.7	5.6	0.9	6.9	2.2
	Set	24.7	24.0	36.9	7.9	11.7	8.5	1.2	6.5	4.0
	Out	17.0	18.0	35.8	8.1	10.7	7.4	0.7	10.4	6.9
	Nov	14.9	13.0	36.2	7.9	9.7	8.9	1.0	8.6	5.3
	Dec	13.9	13.0	36.2	8.2	9.2	10.1	1.1	7.8	5.1
	Jan	12.4	12.0	36.7	8.0	8.9	16.3	1.2	7.2	5.8
	Fev	15.8	15.0	37.0	8.2	9.0	4.1	1.1	7.5	5.1
	Mar	16.0	16.0	36.7	7.9	13.1	5.7	1.2	6.9	1.2
	Mean ± std	17.8±3.8	17.8±4.2	36.4±0.4	8.2±0.2	11.4±2.0	7.7±3.4	1.3±0.6	6.6±2.4	4.8±2.7
GP2	Mar	17.2	16.0	36.2	7.9	9.8	8.6	0.9	1.6	3.7
	Apr	21.7	19.0	35.7	8.0	10.0	7.1	1.3	2.4	2.6
	May	18.5	17.0	36.0	8.3	14.7	7.6	1.4	10.6	13.6
	Jun	19.4	19.0	36.4	8.5	15.5	10.3	2.4	10.5	10.3
	Jul	24.4	25.0	36.2	7.8	9.5	11.1	0.8	11.7	4.3
	Aug	22.7	24.0	36.4	8.1	5.2	9.7	1.5	10.3	5.2
	Set	25.0	23.0	33.0	7.6	5.8	8.5	1.5	12.0	7.5
	Out	17.8	18.0	35.9	8.0	7.2	25.0	0.8	21.3	19.2
	Nov	14.3	13.0	36.1	7.9	8.1	13.2	0.9	5.2	4.2
	Dec	12.5	12.0	36.3	8.2	7.9	11.6	0.7	2.4	3.1
	Jan	15.0	14.0	36.2	8.0	8.7	13.3	2.4	11.9	8.1
	Fev	12.9	10.0	37.1	8.1	8.0	11.5	1.1	15.4	10.8
	Mar	16.6	15.0	36.8	7.9	10.2	10.9	0.9	13.6	8.4
	Mean ± std	18.3±4.2	17.3±4.7	36.0±1.0	8.0±0.2	9.3±3.0	11.4±4.5	1.3±0.6	9.9±5.7	7.8±4.8

Chlorophyll *a* in the sediment varied markedly during the experimental period. In general, throughout the experimental period, GP_2 presented higher levels of chlorophyll *a* in the sediment (annual average – 9.9±5.7 µg g^{-1}) than GP_1 (annual average – 6.6±2.4 µg g^{-1}). In both sites, the minimum values were recorded in March 08 (GP_1 - 0.2 µg g^{-1}; GP_2 - 1.6 µg g^{-1}) and the maximum values in October 08 (GP_1 - 10.4 µg g^{-1}; GP_2 - 21.3 µg g^{-1}). The GP_2 presented significantly higher chlorophyll *a* levels (K-W., H=4.7, *df*=1, *P*=0.031).

The phaeopigments in the sediment showed no significant differences between ground plots (K-W., $P>0.05$). In March 08, no phaeopigments values were recorded at GP_1 and the lowest value at GP_2 was found in April 08 (2.6 µg g^{-1}). The highest phaeopigments levels were observed in May 08 for GP_1 (10.8 µg g^{-1}) and October 08 for GP_2 (19.2 µg g^{-1}).

7.3.2. Mortality

The mortality of clams throughout the experimental period is illustrated in Figure 7.2. The results show a clear increase in the percentage of mortality in the first month of culture, particularly for GP_1 (≈ 40 % at GP_1 and ≈ 20 % at GP_2). This period was designated as Phase I and considered as the adaption stage of organisms to the ground plot. After this phase, the values of mortality at GP_1 presented a slight increase of approximately 13 % throughout the experimental period (Phase II). At GP_2, after Phase I, the mortality percentages continued to increase sharply until late summer (September 08) and then remained almost stable during autumn months, increasing slightly afterwards during the winter months. There were no significant differences between ground plots (K-W., $P>0.05$).

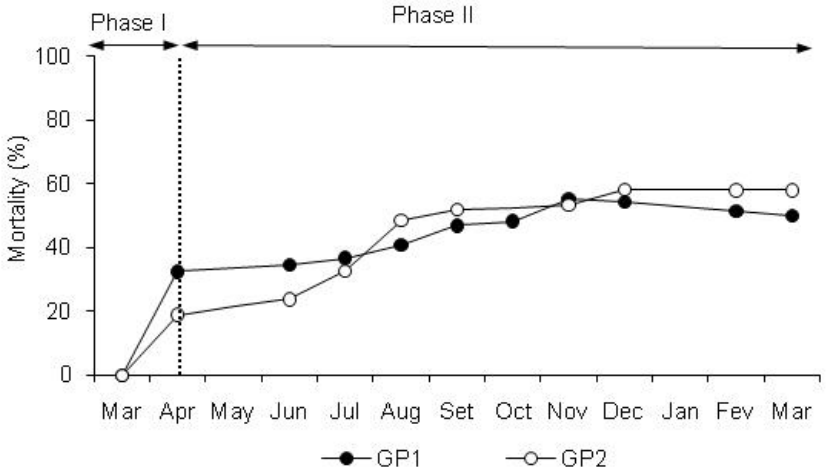


Figure 7.2. Mortality percentage of *Ruditapes decussatus* evaluated in the two ground plots (GP_1 and GP_2) between March 2008 and March 2009.

7.3.3. Growth and allometry

The average monthly values of length, weight and growth rate of the clams in the two ground plots are presented in Table 7.2.

In general, in both sites, there was an increase in the population size (length and weight) throughout the experimental period (annual increase in length - GP_1=10.02 mm and GP_2=8.32 mm; annual increment in weight - GP_1=4.82 g and GP_2=3.66 g). These increases in growth were much more pronounced during the spring/summer seasons and almost stagnated during the autumn/winter ones. The biometric variables, length and weight presented significantly higher values at GP_1 (length: K-W., H=97.1, df=1, P≤0.001; weight: K-W., H=78.2, df=1, P≤0.001), a maximum difference between the two ground plots was observed in July 08 for the length and in August 08 for the weight.

The instantaneous growth rates in length and weight tend to decrease throughout the experimental period in both ground plots. There were no significant differences between ground plots (ANOVA, P>0.05).

Table 7.2. Temporal evolution of growth (length and weight) (mean±SD, n=30) and growth rates of *Ruditapes decussatus* collected in the two ground plots (GP_1 and GP_2) between March 2008 and March 2009.

Ground plots	Month	Lengh (mm)	Lenght growth rate (K)	Weight (g)	Weight growth rate (K)
GP1	Mar	22.11±3.54		2.51±1.25	
	Apr	25.14±2.47	0.0044	3.43±0.93	0.0109
	May	27.12±2.59	0.0035	4.18±1.10	0.0088
	Jun	29.41±3.10	0.0029	5.50±1.58	0.0079
	Jul	30.30±2.67	0.0027	5.72±1.37	0.0071
	Aug	31.80±2.61	0.0025	6.62±1.49	0.0066
	Set	32.45±2.60	0.0022	7.03±1.45	0.0058
	Out	31.72±2.75	0.0016	6.51±1.54	0.0044
	Nov	32.04±3.48	0.0015	7.01±1.62	0.0041
	Dec	31.57±3.42	0.0012	7.02±1.98	0.0035
	Jan	-	-	-	-
	Fev	32.87±2.54	0.0012	7.36±1.69	0.0032
	Mar	32.13±2.18	0.0010	7.33±1.48	0.0029
GP2	Mar	22.11±3.54		2.51±1.25	
	Apr	25.31±2.86	0.0048	3.55±1.08	0.0124
	May	25.47±3.16	0.0024	3.59±1.20	0.0061
	Jun	26.63±3.16	0.0021	4.21±1.34	0.0060
	Jul	26.43±3.22	0.0016	4.49±1.51	0.0051
	Aug	29.06±2.83	0.0019	5.31±1.42	0.0051
	Set	30.57±2.07	0.0018	6.02±1.23	0.0050
	Out	30.72±2.69	0.0015	6.21±1.50	0.0042
	Nov	31.09±2.49	0.0014	6.25±1.34	0.0037
	Dec	30.53±2.58	0.0011	6.32±1.46	0.0032
	Jan	-	-	-	-
	Fev	30.45±3.13	0.0010	6.26±1.89	0.0027
	Mar	30.43±3.30	0.0009	6.16±1.49	0.0025

The annual and seasonal weight-length relationships of *R. decussatus* collected monthly from March 08 to March 09 from both ground plots and the results of analysis of covariance (ANCOVA) using the ground plot of origin as covariate are present in Table 7.3. The annual allometric relationship weight-length followed an exponential pattern. Clams from both sites grew faster in length than in weight throughout the year and the season (negative allometry – $b<2.95$). Except in summer, significant inter ground plots differences were revealed (ANCOVA, $P<0.05$), for an identical length, the weight was higher in the GP_1.

Table 7.3. Allometric relationship weight-length of *Ruditapes decussatus* collected in the two ground plots (GP_1 and GP_2) between March 2008 and March 2009 and inter ground plots comparison.

Period	Ground plots	Weight-lengh allometric relationship	*n*	*r*	*P* value
Spring	GP_1	$W = 38,1 \times 10^{-5} L^{2,82}$	343	0.97	<0.05
	GP2	$W = 51,5 \times 10^{-5} L^{2,72}$	336	0.97	
Summer	GP_1	$W = 44,4 \times 10^{-5} L^{2,77}$	220	0.97	>0.05
	GP2	$W = 100,5 \times 10^{-5} L^{2,54}$	220	0.97	
Autumn	GP_1	$W = 981,3 \times 10^{-5} L^{1,88}$	215	0.81	<0.05
	GP2	$W = 96,6 \times 10^{-5} L^{2,56}$	219	0.89	
Winter	GP_1	$W = 35,6 \times 10^{-5} L^{2,84}$	251	0.97	<0.05
	GP2	$W = 53,5 \times 10^{-5} L^{2,72}$	248	0.94	
Anual	GP_1	$W = 52,6 \times 10^{-5} L^{2,73}$	927	0.96	<0.05
	GP_2	$W = 51,4 \times 10^{-5} L^{2,75}$	919	0.96	

7.3.4. Condition index

The seasonal evolution of the condition index from both studied sites is illustrated in Figure 7.3. The condition index of clams at GP_1 was permanently higher than at GP_2 during all experimental period. Inter ground plots significant differences were observed (K-W., $H=87.7$, $df=1$, $P<0.001$). The condition index values at GP_1 suffered a slight decrease in the first months and subsequently an accentuated increase from April 08 to June 08 (maximum value -

14.0±1.7) was recorded. Then a decrease was observed until February 09 (minimum value - 6.2±0.9) and after that increased in March 09. At GP_2 the condition index also suffered a slight decrease in the first months and then remained at the same level until July 08, with a maximum value (9.9±1.5) observed in August 08. Then a decrease was observed until March 09 (minimum value - 5.1±1.0).

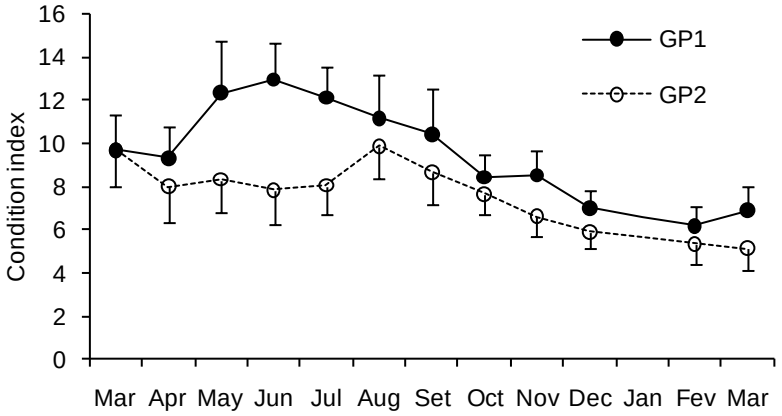


Figure 7.3. Temporal variation of condition index (mean±SD, *n*=25) of *Ruditapes decussatus* collected in the two ground plots (GP_1 and GP_2) between March 2008 and March 2009.

7.3.5. *Biochemical composition*

Variations in the proteins, total lipids and glycogen (main energy reserves for gametogenesis) and total energy contents of clams during the experimental period are presented in Figure 7.4, expressed as a percentage of dry weight (DW) and kilojoules by gram of dry meat weight.

Proteins were the predominant dry tissue constituent of the clams followed by glycogen and total lipids. The highest protein content value was recorded in December 08 (GP_1 – 68.4±1.7 % of DW; GP_2 – 75.5±10.0 % of DW) and the lowest in April 08 (GP_1 – 23.3±1.2 % of DW; GP_2 – 26.8±7.0 % of DW). The annual variation and levels of clam protein content at the two ground plots were very similar, except in July. No significant differences were observed (K-W., P>0.05).

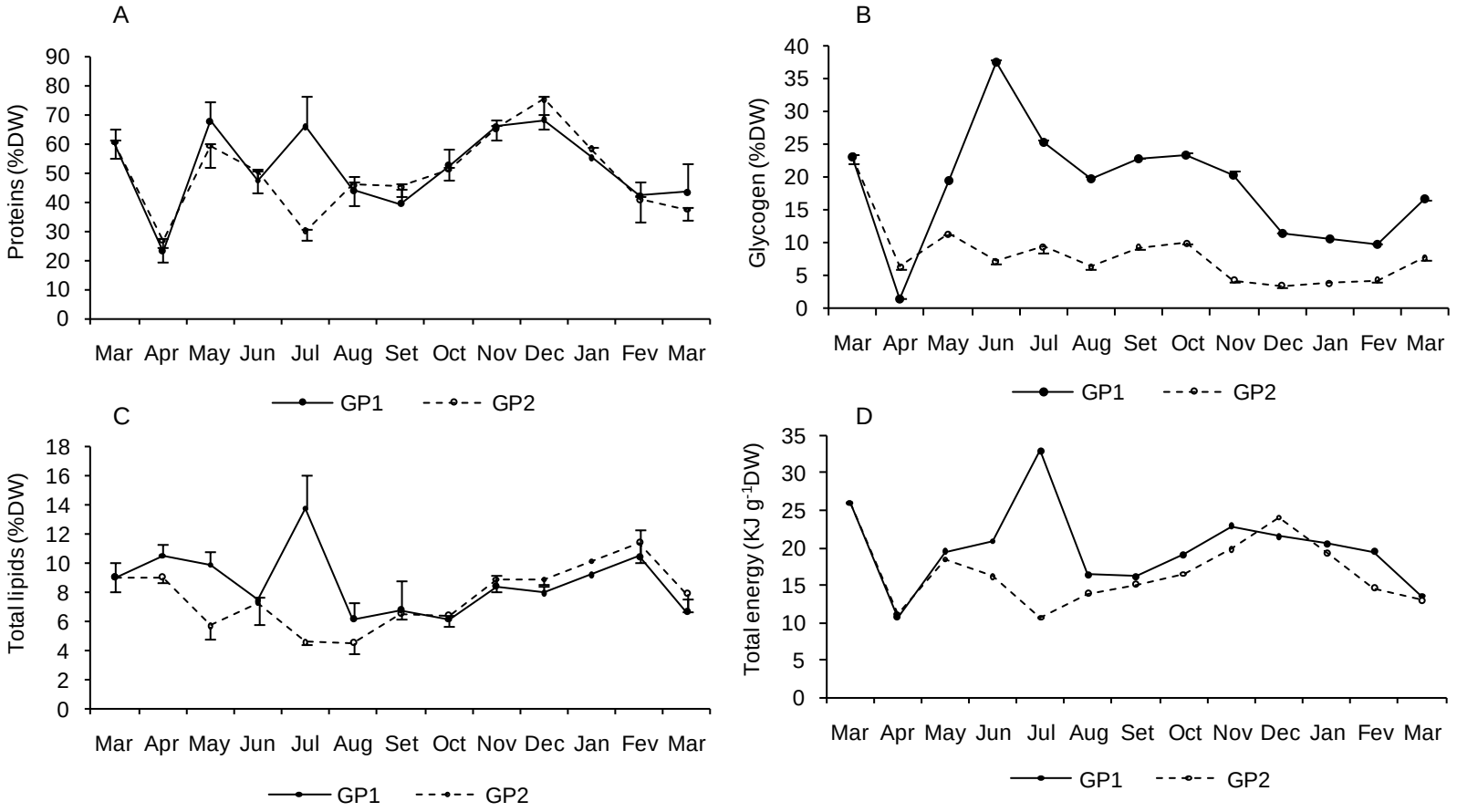
The total lipids content in the clams was significantly different between ground plots, during the spring/summer period the total lipid levels were higher at GP_1 while in the autumn/winter period they were slightly higher at GP_2. Inter ground plots significant differences were recorded (ANOVA, F=9.8, df=1, P=0.002). The lowest (6.1±1.2 % of DW) and the highest (13.6±2.3 % of DW) total lipid values were reached in October 08 and July 08 respectively, at

GP_1. At GP_2, the lowest (4.6±1.1 % of DW) and the highest (11.4±1.4 % of DW) total lipid levels were attained in August 08 and February 08, respectively.

The annual variation of glycogen contents in both ground plots was very similar; however the clams' glycogen levels at GP_1 were permanently higher than at GP_2 after April 08. Inter ground plots significant differences were observed (K-W., H=39.2, df=1, P≤0.001). At GP_1 the glycogen contents of clams suffer a sudden drop at the first month of culture; increased abruptly from April 08 to June 08 to achieving a maximum peak with a consequent decreased until August 08. During the period between September 08 and November 08 it remained practically constant, decreasing in the winter months (December 08 to February 09) with a later increase in March 09. At GP_2, after a sudden drop in the first month of culture similarly to GP_1, glycogen presented an erratic variation until October 08, decreasing in November 08. During the winter months (December 08 to February 09) glycogen remained at a constant level, suffering a subsequent increase in March 09. The lowest glycogen values were observed in April 08 (GP_1 – 1.4±0.2 % of DW) and December 08 (GP_2 – 3.4±0.1 % of DW) and the highest in June 08 (GP_1 – 37.5±0.5 % of DW) and March 08 (GP_2 – 23.5± 1.0 % of DW).

Proteins and total lipids were the constituents that mostly contributed to the total energy content; effectively the annual variation of this component is very similar to the annual variation of proteins. The clams' total energy levels at GP_1 were higher than at GP_2 from May 08 to November 08. Statistically significant differences among sites were observed (K-W., H=16.3, df=1, P≤0.001).

Figure 7.4. Temporal variation (mean±SD, *n*=10) of biochemical composition (A - proteins, B - total lipids and C - glycogen) and total energy (D) in *Ruditapes decussatus* collected in the two ground plots (GP_1 and GP_2) between March 2008 and March 2009, expressed as percentage of dry weight of clams (% DW) and Kilojoules by gram of dry meat (KJ g^{-1} DW).



7.4. Discussion

The results obtained in this study demonstrate the influence of different environmental conditions in the performance of the clam *R. decussatus* culture.

Due to the proximity of GP_2, to the most important local fishing harbour, the presence of high levels of tributyltin (TBT) and polycyclic aromatic hydrocarbons (PHAs) have been reported in clams by Cravo et al. (2012). Contrary GP_1, due to a very low level of surrounding urbanizations, proximity of the sea inlet and high hydrodynamic characteristics was appropriate as a reference site to the comparison of the biological and physiological responses of clams to different environment parameters in this study. In terms of abiotic factors (water and sediment temperature, salinity, pH, dissolved oxygen, particulate organic matter and organic matter, chlorophyll *a* and phaeopigments) evaluated in this study, dissolved oxygen, particulate organic matter, chlorophyll *a* and phaeopigments showed significant differences between the two studied sites.

In shellfish production the organisms' survival is dependent on cumulative mortality, which is often the sum of mortalities originated by various factors (the seed size, biological and physiological condition, environmental pathogens and predation) and the interaction between them. Effectively in this study the mortality rate observed in both ground plots seems to be originated by the interplay of several of these factors, showing a different importance at each ground plot. In fact, at GP_1 during the first months (Phase I) of sampling, a lot of damaged clam shells were observed suggesting that the predation effect was the main cause of mortality. At GP_2 the mortality was essentially associated with the low physiological condition observed during all the sampling period and the low levels of dissolved oxygen observed in the summer period (see Table 7.1 and Figure 7.3).

The growth of bivalves varies with several factors: growing area, season of the year, temperature, quantity and quality of food and dissolved oxygen (Martinez et al., 1997) among others. While many of these factors are dependent on each other, some of them are dominant; indeed food availability and temperature are the factors that have the major influence on growth (*e.g.* Eversole et al., 1986; Robert et al., 1993; Paterson and Nell, 1998). However, when we compare the growth of the clam *R. decussatus* in the two ground plots, the amount of food available and temperature did not seem to have a preponderant effect. Although GP_2 presented significantly higher sediment chlorophyll *a* levels than GP_1, clams presented significantly higher growth in length and weight in GP_1, suggesting that the quality of food is extremely important for the healthy development of bivalves. According to Toro et al. (1999) bivalves spend an extra amount of energy selecting the most suitable food for their development, creating a stress situation in organisms. Differences in allometry allow life-history comparisons between populations from different habitats. Intersite differences observed in weight-length relationships

were related with the growth differences. It must be noted that the shell length and weight of clams from the GP_2 never reached maximum values as high as those registered for GP_1 specimens.

Clams from ground plot GP_1 presented better biological and physiological conditions in terms of condition index and biochemical composition to the cost tolerance endured by organisms from the GP_2 to face to known adverse environmental conditions. The condition index of clams has been considered as the key parameter of the sexual maturation process (*e.g.* Walne and Mann, 1975; Matias et al., 2009) and is usually regarded as a useful tool to evaluate the nutritive status of clams (Crosby and Gale, 1990). This parameter is influenced by many factors including food availability, temperature and most importantly reproduction (Tlili et al., 2012). The clams' condition index at both ground plots was higher in spring/summer seasons, when environmental conditions are favourable, rather than in winter, when food availability is more limited. A condition index decreasing pattern was detected at the end of summer, associated to the fluctuations in the reproductive cycle of *R. decussatus*. In this species, spawning period is coincident with summer, when stressful environmental parameters (high temperatures and low dissolved oxygen) are more notorious.

Several studies on bivalves have shown that gametogenesis is associated with an annual cycle of the accumulation and use of energy reserves, which is influenced by environmental parameters such as food availability and temperature (Fernandez-Castro and Vido de Mattio, 1987; Massapina et al., 1999; Pérez-Camacho et al., 2003; Joaquim et al., 2011). Generally, energy is accumulated when food is abundant, and this energy is then used to synthesize gametes, which are liberated during the spawning process. This cycle translates into a seasonal pattern of biochemical composition that can vary among populations and species (Albentosa et al., 2007; Matias et al., 2009). The relative amounts of proteins (GP_1- 23.3 to 68.4 % of DW; GP_2 – 26.8 to 75.5 % of DW), glycogen (GP_1- 1.4 to 37.5 % of DW; GP_2 – 3.4 to 23.5 % of DW) and total lipids (GP_1- 6.1 to 13.6 % of DW; GP_2 – 4.6 to 11.4 % of DW) measured in *R. decussatus* were similar to those previously described in the literature for this species (*e.g.* Ojea et al., 2004; Aníbal et al., 2011). Many authors have suggested that somatic proteins, beyond being used for structural function, are also used as an energy reserve during gametogenesis in bivalves (Gabbott and Bayne, 1973; Beninger and Lucas, 1984; Liu et al., 2008). In this study, in both sites, the proteins did not show a clear seasonal fluctuation; however there was a net increase in March and May, probably related with the accumulation of gametes. Similarly to the clam *R. philippinarum* (Adachi, 1979), *R. decussatus* seems to mobilize the somatic proteins to obtain energy, since there was a marked decrease from December 08, which is coincident with the onset of gametogenesis. During the spring/summer seasons, the erratic variations in protein content seem to be related with losses due to the successive spawnings. Pérez-Camacho et al. (2003) have showed that lipids constitute the reproductive reserve *par excellence* in *R. decussatus* for gamete maturation and as energy reserves in natural conditions or imposed by nutritional stress, particularly in the autumn/winter

when the water temperatures and food availability decrease. The highest levels of total lipids correspond to spawning periods, reflecting the fact that they constitute, after proteins, a major component of the bivalve oocytes (Holland, 1978, Beninger and Lucas, 1984, Massapina et al., 1999). Nonetheless, carbohydrates have been likewise considered the main source of energy in bivalves (Zwann and Zandee, 1972; Barber and Blake, 1981), in particular for gametogenesis (*e.g.* Barber and Blake, 1985; Martínez, 1991). The temporal relation between glycogen and lipids shows that glycogen reserves are converted into lipid gametes before spawning (Beninger and Lucas, 1984). Several authors have, indeed, reported a maximum of glycogen content in bivalves immediately preceding and during gamete proliferation (Ansell et al., 1980; Barber and Blake, 1985; Ojea et al., 2004). In this study and in both ground plots, during the autumn/winter seasons low values of glycogen and high values of lipids were observed, emphasizing the fact that *R. decussatus* during vitelogenesis uses the stored glycogen to perform *de novo* synthesis of lipids. The accumulation of lipids during the winter months confirms that lipids represent important metabolic reserves to energetic sustain during winter and to gametogenic processes. The comparison of the two ground plots shows that the lipid content between the months of April 08 to August 08 was generally higher in GP_1 reflecting a higher reproductive capacity of clams cultured in this ground plot. The glycogen content showed the same trend, but throughout all the sampling year, which reflected the better environmental conditions in this ground plot. This fact was also supported by the observed values of condition index and the total energy content.

The decrease in clams energy content observed in both ground plots at July/August 08 was mainly due to successive spawnings, while the decrease observed in winter (more pronounced in the GP_2) was mainly due to the food availability (lower values of chlorophyll *a*).

The physiological and biological condition of clams were significantly higher in GP_1 suggesting a poorer nutritive status of clams in GP_2. However GP_2 presented a greater availability of food (significantly higher chlorophyll *a* in sediment) and higher temperatures. These contradictory observations suggest that the quality of food was more important than the quantity and/or that the effect of the existent contaminants in GP_2, for example contaminants acting as endocrine disruptors, were higher than the effect of food availability and temperature on clams physiological processes. Effectively, TBT may affect the mixed function oxygenase system that plays a key role in the metabolism of xenobiotics (*e.g.* PAHs), as well as endogenous compounds such as fatty acids and hormones, therefore interactions with this system may affect animal's reproductive competence (Cravo et al., 2012). The spawning period of *R. decussatus* occurs in the spring/summer months and is characterized by a decrease in the condition index and total lipids due the loss of weight/body mass. These patterns were also more evident in clams cultured in GP_1, suggesting once more that the reproductive capacity of clams in GP_2 was sharply reduced.

If we try to link growth and biochemical composition, mainly energy reserves, it can be hypothesized that for *R. decussatus* from GP_1 - reference site, the energy reserves were mainly allocated to gametogenesis and growth, whilst in GP_2 polluted site, part of the energy reserves were used to respond to the chemical stress induced by the presence of pollutants and growth. Consistent differences have indeed been observed in *R. decussatus* biology and physiology between the two ground plots. The most important difference was the reduction of the reproductive capacity in GP_2 that could influence the viability of the culture due to recruitment failure.

Surprisingly, Cravo et al. (2012) had suggested that clams at GP_2 seemed healthier and less stressed than in other studied sites, based on Integrated Biomarkers Response (metallothionin, δ-aminolevulinic acid dehydratase, acetylcholinesterase, lipid peroxidation, DNA demage and alkali-labile phosphate in males and females) and Health Status indices that rank sampling sites and reflect contamination gradients, providing decision tolls to evaluate the ecosystem "health", despite the higher levels of TBTs and PAHs. However these authors' have also highlighted the fact that in ecosystems where pollutants occur in mixtures, such as in the Ria Formosa Lagoon, the relationship between biomarkers and contaminants is often not easily understood. Indeed the interactions among contaminants and biological systems will influence the effect of the environmental compounds on the organisms' response.

The use of a multibiomarker approach in risk assessment seems then essential with the aim of to have a complete knowledge of basal biomarkers response and its seasonal variation to distinguish pollution induced effects from those induced by natural biological cycles of clams, including reproduction (Bocchetti et al., 2008; Cravo et al., 2012). Studies like the present one seem then essential in order to evaluate and highlight the significance of the contaminants on biota biology and physiology. In conclusion, the knowledge of the organisms' mortality and growth, and changes in biochemical composition, especially in energetic reserves cycles (glycogen and total lipids) as a reflection of reproduction, will allow a better understanding of environmental contaminants impacts and should be part of multibiomarker studies in risk assessment. The results obtained in this study could also be useful to design a geographic information system with the better and worst areas for clam production, contributing to provide adequate management strategies.

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Chapter 8

Conclusions

8.1. General Conclusions

Bivalves are the main component of the benthic fauna of many marine and estuarine areas. In the last centuries, due their high economic importance, there has been an on growing bivalve production by man tended to cultivate them. However, until now most of the bivalve production in Europe still relies on the collection of wild seed. Consequently this production is highly dependent of overall environmental conditions that influence growth, survival and reproduction, and ultimately juvenile natural recruitment, the base of the production. This is the case of the European clam *Ruditapes decussatus*, a species with a high social and economic importance in many European countries (mainly in Portugal, Italy and Spain). In order to overcome the random availability of seed, the development of *R. decussatus* rearing programs, and hatchery technology for the production of this species seems essential. However until know only very few studies have been performed on this subject (Fernandez-Reiriz et al., 1999; Albentosa et al., 1996, 1999; Delgado and Pérez-Camacho, 2002, 2005, 2007; Ojea et al., 2004; Ojea et al., 2008).

R. decussatus seed production relies on basic principles that follow successive biological steps (Gosling 2002, Helm et al., 2004, Joaquim et al., 2008). The present work aimed to evaluate relevant biological and ecological processes at different culture phases (broodstock conditioning, larval culture and on-growing). Overall, the ultimate objective of this study was then to create the bases to develop production programs of this species.

Understanding the natural reproductive cycle of bivalve is essential for establishing successful hatchery-based production (Joaquim et al., 2011). The reproductive dynamics and gametogenic cycle of the two main Portuguese populations of *R. decussatus* (Ria Formosa Lagoon and Ria Aveiro) were studied through microscopic analyses of male and female gonads and patterns of nutrient storage and utilization (Chapter 2). The reproductive cycle of *R. decussatus* from both populations followed an annual cyclicality and comprised a resting phase during the period of coldest seawater temperatures (October to January), a ripe stage in spring followed by an extensive spawning period that began in late spring and extend during summer until early autumn. This extended and continuous spawning period, during which occurs successive and simultaneous production of gamets and spawning may be an advantageous strategy for the species because it ensures a continuous supply of gametes. *R. decussatus* can adopt different reproductive strategies depending on the geographic origins. The convergent results of cycle of nutrients stored and utilization showed that clams of both populations present a high reproductive effort that almost depletes its energy reserves; nevertheless, while Ria de Aveiro population retrieves it immediately after spawning, the same is not verified in clams from Ria Formosa Lagoon with their consequent debilitation. Also, based on the glycogen pattern it was possible to infer that the Ria Aveiro population are opportunistic, while the Ria Formosa Lagoon population exhibited an intermediate stategy. Nevertheless, both populations presented viable broodstock for intensive hatchery production of juveniles and the extended spawning

period of both *R. decussatus* populations has interesting implications for the implementation of profitable aquaculture. Moreover, this species presented a great capacity for gonadal regeneration, which coupled with its high gonadal development rate would provide larvae during most of the year without extensive and expensive broodstock conditioning. This information on the gametogenic and spawning periods and consequent energy storage can also provide the optimal reproductive time for artificial spawning induction in aquaculture.

Broodstock conditioning is the first phase in hatchery husbandry essential to obtain larvae for culture (Gosling, 2002). Artificial reproduction of bivalves requires the use of animals that have reached optimal sexual condition, which, according to Kennedy et al. (1996), depends on the synergistic effects of both internal and external factors. However the response of bivalves to conditioning regimes varies widely among species and there are also evidences that the responses can vary, among different geographical populations, in the same species (Iglesias et al., 1996; Avendaño and Le Pennec, 1997). Effectively, the ecotype of *R. decussatus* living in different areas could strongly differ in terms of their fecundity levels and biochemical composition (Shaffee and Daoudi, 1991; Trigui-El-Menif et al., 1995). The determination of the effect of geographic origin, temperature and timing of broodstock collection on conditioning, spawning success and larval viability of *R. decussatus* provided detailed information on the response of two different populations to different conditioning variables (Chapter 3), crucial to hatchery production. Of the three variable analysed (broodstock origin: Ria Formosa lagoon and Rias Galegas; conditioning temperatures: 18±1ºC, 20±1ºC and 22±1ºC and timing of broodstock collection: October and February), the timing of broodstock collection was the most important determining factor on gametogenic development, spawning and larval rearing. Geographic origin and conditioning temperature also greatly affected the spawning. In conclusion, the February conditioning was more effective than the October one and the best conditioning temperatures were 20±1ºC and 22±1ºC for North and South populations, respectively; the efficient conditioning temperature for each population showed to be related to the seasonal temperature regime in their geographic origin. Larval viability and growth performance seemed to be independent of the broodstoock conditioning.

The manipulation of the gonadal cycle and spawning period of the clams so that adults can spawn earlier or later than occurs in the natural environment is indeed crucial (Ojea et al., 2004). As mentioned earlier in broodstock conditioning temperature and food availability are key external factors. The biochemical composition of the diet influences the physiology of bivalves. In general, changes in each biochemical component are closely linked to the state of sexual maturity of the bivalve, and are related to energy supply either directly from the ingested food or from previously stored reserves (Navarro et al., 2000; Pérez-Camacho et al., 2003). The evaluation of effect of different diets and temperatures on the biochemical composition during sexual maturation and spawning success provided detailed information on hatchery broodstock performance of *R. decussatus* (Chapter 4). This result showed that nutritional value of the diet supplied to broodstock during conditioning clearly influences the gametogenesis process, energy storage and spawning success, as was evidenced by the differences in the reproductive effort between the monospecific

(*Isocrhysis aff galbana* and *Chaetoceros calcitrans*) and bispecific (*I. aff galbana* plus *C. calcitrans*) supplemental diets tested. Moreover it was also evident that temperature is a parameter which must be carefully managed to improve the reproductive output, indeed although the highest temperature throughout gametogenesis shortened the time to full ripeness, it did not produce a better reproductive output. It was also shown that *R. decussatus* lipids contents, especially triacylglycerols, were clearly of major importance in conditioning, either as energy reserves or as precursors of tissue structures. In conclusion, these observations should be taken into consideration in *R. decussatus* production programs for broodstock conditioning and consequently production of high quality spat and can contribute to improve global hatchery technological development of *R. decussatus*.

Larval rearing is the second phase in hatchery husbandry and its initial) success is closely related to the preceding step. Successful bivalve larval growth and development depends on the energy available during the endotrophic and the subsequent exotrophic developmental phases (Labarta et al., 1999; Pernet et al., 2004). Moreover during the early endotrophic and mixotrophic phases, larvae rely essentially on existing reserves from the female gametes (Labarta et al., 1999). The subsequent exotrophic phase that lasts until larval metamorphosis depends on the value of the diet provided to promote larval growth (Whyte et al., 1990). The major morphological changes in bivalve development occur during embryogenesis and metamorphosis (Bayne et al., 1975). The duration of these periods is species specific and strongly dependent on rearing temperature and food (Rico-Villa et al., 2009). The *R. decussatus* duration of the endotrophic/exotrophic transition period and the selective use of different biochemical substrates in both absolute and energetic terms during larval development were studied thought comparison between reared fed and unfed larvae (Chapter 6). This comparison between fed and unfed larvae highlighted the importance of egg lipid reserves, especially neutral lipids, during the endotrophic phase of embryonic development (first two days after fertilization). Egg reserves, however, also showed to contribute energetically to the maintenance of larvae beyond the embryonic development. Effectively, in fed larvae, the endotrophic phase was followed by a mixotrophic phase extending to days 5-8 after fertilization, and a subsequent exotrophic phase. Metamorphosis showed to start around day 20. The intense embryonic activities during this phase were supported by energy derived from lipids, mainly from neutral lipids and the metamorphic activities were supported by energy derived essentially from proteins accumulated during the planktonic phase and depend on the nutritional value of diets. In conclusion, The results obtained give us an important general knowledge of the biochemical dynamics in the early developmental phases, suggesting that the monospecific diet *I. aff galbana* seems to be more adequate than *C. calcitrans* and that food supply should be rather moderate until day 8 (mixotrophic phase) to decrease the impact of contamination source. These conclusions provide important information for a successful larval production of *R. decussatus* hatchery programs.

Although, the larval stages are presently thought to be the most critical stages in the life cycle of bivalves, some biological aspects are still poorly known (Helm et al., 2004; Rico-Villa et al., 2006). A key limiting factor at this stage concerns indeed our limited knowledge of larval feeding requirements; this is particularly true for *R. decussatus*. It was then essential to this identify feeding regimes that result in maximum growth, survival and settlement but which in turn also reduce hatchery operating costs. The effect of the nutritional value of two different common microalgae used in bivalve hatcheries, *I. aff galbana* and *C. calcitrans* used as monospecific and bispecific diet in different proportions, on the survival, growth, settlement and biochemical composition of *R. decussatus* larvae was evaluated, allowing the obtention of a basic, but crucial information of nutritional requirements for the first time (Chapter 7). This study clearly showed that *R. decussatus* larvae cannot use *C. calcitrans* with the same efficiency than *I. aff galbana* at early stages of development; however the inclusion of *C. calcitrans* improved late larval development. In conclusion, and based on a holistic approach to achieve optimal larval growth, we could suggest an optimal larval feeding plan: raise larvae with *I. aff galbana* monospecific diet during a first period (2-5 days after fertilization), followed by the bispecific diet *I. aff galbana* plus *C. calcitrans* with the flagellate in most proportion (5-17days after fertilization) and then during late larval period *I. aff galbana* plus *C. calcitrans* with the diatom in the higher proportion. The establishment of the most adequate nutritional regime and ratio at each larval development stage is crucial information to define *R. decussatus* larval rearing programs.

The on-growing phase is the ultimate phase of the production and aims to grow seeds to commercial size as quickly as possible to make the operation economically attractive. However, it is also a problematic step due to the fact that it is mostly dependent of the natural environmental condition. Effectively, highly productive areas such as estuaries and coastal lagoons are often at risk due to increasing stress from anthropogenic activities such as urbanization, industrialization, intensive agriculture and mass tourism. Complex mixtures of contaminants are continuously released in these systems deteriorating the water quality and imposing severe restrictions to organisms and possibly causing a decrease in natural resources (Monserrat et al., 2007; Cravo et al., 2009; Cravo et al., 2012; Tlili et al., 2012). An example is the Ria Formosa Lagoon, a major coastal lagoon in the Portuguese south coast. *R. decussatus* is extensively produced and harvested in the Ria Formosa Lagoon, where clam farming represents an important economical sector. The environmental quality assessment of clams' ground plots requires the evaluation of integrated biological effects. Experimental *R. decussatus* on-growing surveys on two clams' ground plots of the Ria Formosa Lagoon with different environmental conditions (one in the proximity of pollution sources and with low water renewal and other more clean and with high water renewal) was performed in order to evaluate the impact of environment on the biological and physiological status of the individuals (Chapter 8). The results showed that organisms' mortality, growth, and changes in biochemical composition, especially in energetic reserves cycles (glycogen and total lipids), as reflection of reproduction gives indeed a good knowledge of the impact of the environment on the organisms. The analysis

of these biological and physiological parameters clearly demonstrated a different performance of *R. decussatus* between the two ground plots. The most polluted ground plot showed a reduction of the reproductive capacity that consequently could influence the viability of the culture by causing recruitment failures. The results obtained in this study, support the potential use of this kind of study to design geographic information systems identifying the better and worst productive clam areas, contributing, this way, to provide adequate management strategies.

As previously mentioned the ultimate objective of the present work was to create biological and ecological bases to develop *R. decussatus* production programs. The overall information gathered in this study will allow the improvement of all the phases of *R. decussatus* production programs constituting this way an important step in the European clam production and a prerequisite for future works.

8.2. Final Remarks and Perspectives

The present study allowed the improvement of the knowledge available on bivalve production in general and particularly the scarce information on all phases of *R. decussatus*, with emphasis on some relevant aspects of biology and ecology. Furthermore, some of the experiments performed provided the first information available on the reproductive dynamics and gametogenic cycle for the two most important Portuguese populations of *R. decussatus* as well as the data available on this species nutritional requirements (particularly in terms of broodstock conditioning and larval development) and also on the impact of the environmental conditions on this species biology and physiology (namely mortality, growth and reproduction capacity). In addition, in all studies, particular emphasis was given to the application and/or development of methodologies that could be routinely applied to *R. decussatus* production.

As in any research work some questions require further studies and new topics to be addressed in future studies arose. The present study highlighted indeed the need for continuous research studies linking fundamental and applied approaches to examine the complex biological processes and provide innovative technology in order to improve *R. decussatus* seed production.

Future studies should focus on the evaluation of the influence of other microalgae diet in broodstock conditioning, namely locally-isolated phytoplankton diets, aiming to optimize this culture phase. Furthermore, in this culture phase one of the most remaining important bottlenecks is the spawning success and oocyte quality, in this way the performance of multidisciplinary studies with zoothecnical, genomic and proteomic approaches that will allow the establishment of a consistent methodology to obtain gamete with high quality seems essential.

Additionally, the baseline data gathered on larval culture phase, mainly the knowledge of the specific importance of the lipids and proteins during larval development highlights the

need to develop further research on potential algal species or inert diet formulae that would supply the requirements of the larvae in term of biochemical composition, especially in lipids contents. Also, the role of the different lipid classes and fatty acid profiles during larval development should be address to reinforce the larval nutritional requirements knowledge.

This study has also drawn the attention to some features of the effect of different environmental conditions on the clams' response in the on-growing phase. Managing the clam on-growing phase requires a clear knowledge and integration of local environmental characteristic and resources. In fact, it is necessary to develop further studies in order to improve good practice management, such as the evaluation of the most adequate substrate, density culture and protection to predators. Moreover, regular monitoring of ground plot should be made for the detection of pathologies and parasites on clams. In fact, Perkinsus *olseni* is a parasite of *R. decussatus* with a high level of virulence that in conjugation with adverse environmental condition and with a biologic debility of the organism can cause high mortalities. However, most of the studies developed until now mainly focused on the parasite (*e.g.* Vilas et al., 2011; Costa and Costa, 2012; Fernandez et al., 2012), and it is however important to extend this studies on a concise effect of the parasite on biology and physiology of organism aiming to predict and avoid mortalities. This information could be also useful in future selective production programs. Those programs should rely on a holistic approach of environmental conditions and production technologies.

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